Genotype-phenotype relations in lupus nephritis

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Abstract

This thesis explores genotype-phenotype relations in lupus nephritis. Over the past decade, advances in genetic research have improved our understanding of the genes that contribute to lupus susceptibility. However a clear understanding of how these genetic factors interact with the immune system to produce disease in a given patient or group of patients with SLE is difficult to define at this time. Recent monogenic causes of SLE have provided valuable insights into lupus pathogenesis.

A comprehensive clinical characterisation of 164 biopsy proven lupus nephritis patients was undertaken in the initial phase of this research. 27% of patients recruited were of juvenile onset, as defined by diagnosis of nephritis before 18 years of age. Non-European lupus nephritis patients had a younger age of onset and higher rate of progression to end-stage renal disease. Sixteen individuals had a first degree family history of lupus nephritis including five sibling pairs. Familial clustering of nephritis was associated with juvenile onset disease and a more severe clinical phenotype.

The entire cohort was genotyped by ImmunoChip for known lupus susceptibility polymorphisms. Associations with lupus nephritis were found in polymorphisms in the HLA region, *IRF5*, *ITGAM*, *STAT4*, *TNFAIP3*, *TNFSF4* and *ETS1*. Whole Exome Sequencing of familial lupus nephritis cases identified a large number of potential candidates for functional investigation. A promising short list of candidates was created using pedigree information, focusing on variants predicted to be damaging and by incorporating prior knowledge of the biologic pathways implicated in lupus. Candidates under consideration for functional testing include genes involved in activation of Ras pathways, genes involved in the antioxidant defence system, variants in ubiquitination pathways, mutations in serine/threonine protein kinases and genes involved in toll-like receptor and type I interferon signalling pathways.

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I dedicate this thesis to my husband and son, Colin and Conor Mason.

Declaration

I declare that I have personally prepared this thesis and that the work included here is my own, unless otherwise stated. All external information sources are referenced accordingly.

Natasha Jordan

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List of Abbreviations

1KG: 1,000 genomes project

ACR: American College of Rheumatology

AGS: Aicardi-Goutieres syndrome (AGS)

AI: activity index

ANA: anti-nuclear antibody

ANCA: anti-neutrophil cytoplasmic antibody

Anti-dsDNA: anti-double stranded DNA antibody

Anti-RNP: anti-ribonucleoprotein antibody

Anti-Sm: anti-Smith

APS: Antiphospholipid syndrome

aCL: anti-cardiolipin antibody

ASRED: allele-specific restriction enzyme digestion

BILAG: British Isles Lupus Assessment Group

BLys: B-Lymphocyte stimulator

CASQ: clinical activity with serological quiescence

cGRS: count genetic risk score

CHD: Chromodomain helicase DNA binding

CI: chronicity index

CIE: counterimmunoelectrophoresis

CNS: central nervous system

CNV: copy number variation

CSF: cerebrospinal fluid

CD/CV: Common Disease Common Variable

EMEA: European Medicines Evaluation Agency

eQTL**:** expression quantitative trait loci

ESRD: end-stage renal disease

EVS: Exome Variant Server

FcγR: Fc gamma receptors

FDA: Food and Drug Administration

FILS: facial dysmorphism, immunodeficiency, livedo, and short stature

GDI: Gene Damage Index

GFR: glomerular filtration rate

GSH: glutathione

GWAS: genome wide association studies

HRT: hormone replacement therapy

IBS: identity by state

ISN/RPS: International Society of Nephrology/Renal Pathology Society

IRS: immunoregulatory sequences

LA: lupus anticoagulant

LD: linkage disequilibrium

MAF: minor allele frequency

MDS: multidimensional scaling

MSMD: Mendelian Susceptibility to Mycobacterial Disease

NETs: Neutrophil extracellular traps

NGS: next generation sequencing

NIH: National Institutes of Health

NKF KDOQI: National Kidney Foundation Kidney Disease Outcomes Quality Initiative

OD: odds ratio

PAMP: pathogen-associated molecular patterns

PBMC: peripheral blood mononuclear cell

pDC: plasmacytoid dendritic cell

PGA: physician's global assessment

PolyPhen: Polymorphism Phenotyping

PRR: pattern recognition receptors

RNA-Seq: RNA sequencing

SACQ: serologically active but clinically quiescent

SAVI: STING-associated vasculopathy with onset in infancy

SHD: Slp homology domain

SIFT: Sorting Intolerant From Tolerant

SLE: Systemic Lupus Erythematosus

SLEDAI: Systemic Lupus Erythematosus Disease Activity Index

SLICC: Systemic Lupus International Collaborating Clinics

SLPs: Synaptotagmin-like proteins

SNP: single nucleotide polymorphism

SPENCD: spondyloenchondroplasia

SRI: SLE Responder Index

STING (stimulator of interferon genes)

TLR: toll-like receptor

uPCR: urinary protein-creatinine ratio

WES: whole exome sequencing

wGRS: weighted genetic risk score

Yaa: Y-linked autoimmune acceleration

Chapter 1

Introduction

The purpose of this chapter is to:

1. Provide an overview of SLE, diagnosis, epidemiology, classification criteria, assessment of disease activity and damage, treatment and outcomes.
2. Describe the role of genetics in our understanding of SLE from Genome Wide Association Studies and monogenic lupus.
3. Discuss important factors in the pathogenesis of lupus nephritis including the roles of complement, apoptosis, autoantibodies and type I interferon pathways.
4. Introduce the role of monocytes in lupus nephritis providing evidence from murine models and human studies.
5. Set out the aims and objectives of this thesis.

1.1 Introduction to Systemic Lupus Erythematosus

1.1.1 Systemic Lupus Erythematosus - a clinically heterogeneous disease

Systemic Lupus Erythematosus (SLE) is considered the prototypic autoimmune disease with aberrations throughout the immune system resulting in diverse clinical manifestations. SLE is a heterogeneous disease and no single clinical feature or laboratory test is diagnostic of the disease.

The American College of Rheumatology (ACR) classification of SLE requires that a patient have 4 of 11 criteria to be classified as having SLE (Hochberg et al., 1997). These cardinal features include malar rash, discoid rash, photosensitivity, mucosal ulcers, serositis, arthritis, renal abnormalities, neurological disorders, haematological and immunological abnormalities and a positive ANA (anti-nuclear) antibody. The Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for SLE were recently introduced and place more emphasis on renal and immunologic parameters and less on dermatological features than the previous ACR criteria. In the SLICC classification, 4 criteria are required for diagnosis, including at least one clinical criterion and one immunologic criterion or the patient may be considered to have SLE if they have biopsy-proven lupus nephritis in the presence of ANA or anti-double-stranded DNA antibodies (Petri et al., 2012).

1.1.2 Lupus nephritis; a severe manifestation of SLE

Lupus nephritis and CNS (central nervous system) lupus are considered to be at the severe end of the spectrum of clinical manifestations of SLE. Approximately 50% of SLE patients develop nephritis during their disease course, generally in the earlier stages of disease. Lupus nephritis is divided histologically into 6 subclasses as per the ISN/RPS (International Society of Nephrology/Renal Pathology Society)

classification. These classes encompass mesangial disease (classes I and II), proliferative nephritis (classes III and IV), membranous nephritis (class V) and glomerulosclerosis (class VI) (Weening et al., 2004). An estimated 10-15% of lupus nephritis patients progress to end-stage renal disease (ESRD) and require supportive therapy such as dialysis or renal transplantation (Cervera et al., 2003; Mavragani et al., 2003; Adler et al., 2006). Recurrent flares of lupus nephritis activity are associated with poor long-term renal outcomes (Moroni et al., 1996; Mosca et al., 2002). Renal damage is the overall most important predictor of mortality in SLE patients (Danila et al., 2009).

Treatment of lupus nephritis is comprised of two phases; induction and maintenance. The aim of induction therapy is to achieve a meaningful renal response with reduction in proteinuria and preservation of renal function. The goal of maintenance therapy is consolidate the renal response and prevent flares of disease with eventual gradual drug titration. Immunosuppressive treatment should ideally be guided by renal biopsy as clinical, serological or laboratory tests cannot accurately predict underlying renal biopsy inflammation.

1.1.3 SLE prevalence and severity vary with patient ancestry

The prevalence of SLE ranges from 40 to 200 per 100,000 of population, varying considerably in different ancestral groups. SLE is more common in African, Asian and Hispanic populations than in those of European ancestry (Johnson et al., 1995; Feldman et al., 2013; Lim et al., 2014; Somers et al., 2014).

In addition to being more prevalent, the clinical phenotype of SLE is usually more severe in non-Europeans. SLE patients of African ancestry have an increased frequency of lupus nephritis and more damage accrual than Caucasian patients

(Dooley et al., 1997; Alarcon et al., 1999; Hopkinson et al., 2000; Alarcon et al., 2001). SLE patients of East Asian ancestry have younger onset disease with a higher frequency of renal and CNS involvement (Thumboo et al., 2001; Johnson et al., 2006; Peschken et al., 2009). European ancestry has actually been found to be protective against development of nephritis in SLE (Richman et al., 2012).

1.1.4 Female preponderance of SLE

90% of adult onset SLE patients are female. In juvenile onset disease, the female predominance is less pronounced (Hiraki et al., 2008; Mina et al., 2010; Watson et al., 2012). Given the peak incidence of SLE in females in the child bearing years, decreased incidence after the menopause and the increased rate of flare during pregnancy and with use of oral contraceptives and HRT (hormone replacement therapy), a role has been proposed for female sex hormones in SLE pathogenesis. It remains unclear how female hormones could influence SLE pathogenesis. Clinical trials of hormone therapies have been disappointing (Chang et al., 2002). The X chromosome could potentially contribute to lupus aetiopathogenesis independent of sex hormones. The prevalence of Klinefelter's syndrome (47, XXY) is 14-fold higher in males with SLE as compared to those without (Scofield et al., 2008). Genetic modification of the number of X chromosomes carried by mice, XX or XO in females and XY or XXY in males leads to an increased incidence of SLE in those carrying two X chromosomes (Smith-Bouvier et al., 2008).

1.1.5 Morbidity and mortality in SLE

A bimodal pattern of mortality is well recognised in SLE. In the early stages, deaths are caused by intractable lupus activity or infection. In later stages, atherosclerotic manifestations such as myocardial infarction and strokes are common causes of

death (Urowitz et al., 1976; Gladman et al., 2007). The mortality associated with SLE has lessened over recent decades due to earlier diagnosis, improved treatment with immunosuppressive agents and advances in dialysis and renal transplantation. Survival has improved from a 5-year survival of 50% in the 1950s to a 15-year survival of 80% in the 1990s (Merrell et al., 1955; Abu-Shakra et al., 1995). A retrospective review of lupus nephritis patients in the UK over a 30 year period (1975-2005) showed that 5-year mortality decreased by 60% between the first and second decades of the study but has remained unchanged over the third decade. This suggests that the benefits of currently available immunosuppressive therapies have been maximized and that if further advances in SLE outcomes are to be achieved, novel therapeutic targets must be developed (Croca et al., 2011).

1.1.6 Measures of SLE disease activity

A number of indices have been developed to measure SLE disease activity in clinical practice and for research purposes. The most commonly used indices are the British Isles Lupus Activity Assessment Group index (BILAG) and the Systemic Lupus Erythematosus Activity Index (SLEDAI). The BILAG-2004 index is a composite clinical disease activity score, developed for the assessment of SLE disease activity on the principle of the physician's intention to treat (Yee et al., 2009). Neither of these instruments are ideal, therefore a disease activity index combining the BILAG score, the SLEDAI score and a PGA (physician's global assessment) was formulated and is known as the SLE Responder Index (SRI) (Furie et al., 2009). Another important clinical parameter in SLE is quantification of cumulative organ damage and is most commonly assessed by the SLICC Damage Index (Gladman et al., 1996).

1.1.7 Current and emerging therapeutic options in SLE

Conventional immunosuppressive therapies including corticosteroids, cyclophosphamide, mycophenolate mofetil and azathioprine have significantly improved patient survival in SLE over recent decades. Their use, however, is associated with considerable toxicity while a substantial proportion of SLE patients remain refractory to treatment. Recent advances in the understanding of SLE immunopathogenesis have led to the development of more targeted agents. Some of the agents are in advanced stages of development or have entered the realm of clinical practice (such as rituximab and belimumab) while others are in far earlier phases of development.

Treatment of lupus nephritis is comprised of two phases; induction and maintenance. The aim of induction therapy is to achieve a meaningful renal response with reduction in proteinuria and preservation of renal function. The goal of maintenance therapy is consolidate the renal response and prevent flares of disease with eventual gradual drug titration. Immunosuppressive treatment should ideally be guided by renal biopsy as clinical, serological or laboratory tests cannot accurately predict underlying renal biopsy inflammation.

B cell depletion therapy has emerged as a viable therapeutic option in the management of SLE. The main B cell depleting drug used in current clinical practice is rituximab, a chimeric anti-CD20 monoclonal antibody. Rituximab is widely used in SLE but remains unlicensed. Initial open label trials of rituximab in SLE showed encouraging results (Leandro et al., 2002; Looney et al., 2004; Leandro et al., 2005). Disappointing, randomised controlled trial in non-renal SLE (EXPLORER) and lupus nephritis (LUNAR) failed to meet their primary end-points (Merrill et al., 2010; Rovin et al., 2010). More recently corticosteroid sparing regimens in lupus

nephritis have been introduced, with rituximab as induction therapy and a large randomised controlled trial is ongoing (Condon et al., 2013).

Other B cell depleting therapies in development include epratuzumab, an anti-CD22 monoclonal antibody and ocrelizumab, a fully humanized anti-CD20 monoclonal antibody. The B cell survival factor BLys (B-Lymphocyte stimulator) has also been successfully targeted in clinical trials (BLISS-52 and BLISS-76 trials) (Furie et al., 2011; Navarra et al., 2011). In 2011, the United States Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) licensed belimumab, a monoclonal antibody against BLys, as the first new drug in over 50 years for SLE. Belimumab is licensed for use in autoantibody-positive SLE patients excluding those with active lupus nephritis and central nervous system manifestations of SLE. Clinical trials in lupus nephritis are currently ongoing.

Abatacept, a fusion protein consisting of CTLA-4 combined with the Fc portion of human IgG1 (CTLA-4-Ig) is in the advanced stages of clinical trials in SLE. This therapeutic agent blocks the co-stimulatory interactions between T and B lymphocytes can induce immunological tolerance. Therapies targeting cytokines in clinical trials of SLE include tocilizumab, a monoclonal antibody against Interleukin-6, and monoclonal antibodies against interferon-α, sifalimumab and rontalizumab.

The future of SLE treatment looks increasingly promising with the advent of ever more targeted and focused therapies that have emanated from the results of basic science and genetic research. With this in mind, I will now outline the current knowledge of SLE pathogenesis discussing the contributory roles of autoantibodies, complement pathways, defects in apoptosis, toll-like receptors (TLRs) and type I interferon signalling. I will also discuss the role of monocytes in the pathogenesis of

lupus nephritis outlining the growing body of literature supporting their role in the development of the disease.

1.2 SLE - a genetic disease

Epidemiologic studies suggest a significant contribution of genetic factors in the aetiology of SLE. Disease concordance in SLE is higher in monozygotic twins (25-50%) than in dizygotic twins (2%) and there is a high sibling risk ratio (λs) of 20-29 (Block et al., 1975; Deapen et al., 1992; Alarcon-Segovia et al., 2005).

Linkage analysis, candidate gene studies and genome wide-association studies (GWAS) have deepened our understanding of the genetics factors underlying SLE and have led to the identification of several candidate genes. Monogenic causes of SLE are rare but have provided very useful insights into disease pathogenesis as exemplified by mutations discovered in *DNASE1*, *TREX1*, *PRCKD* and complement pathways. It is hoped that the knowledge of lupus genetics will expand significantly with the increasing availability of next generation sequencing (NGS).

1.2.1 Susceptibility genes identified in GWAS

The CD/CV (Common Disease Common Variable) hypothesis predicts that common disease-causing alleles will be found in human populations that harbor a given disease and has provided the theoretical basis for the development of GWAS (Reich et al., 2001; Pritchard et al., 2002; Botstein et al., 2003). GWAS are essentially large case-control studies were all participants are genotyped for the majority of common known SNPs. For each SNP, the allele frequency is compared between cases and controls using Pearson's Chi-squared ($\chi 2$) test allowing odds ratios and *p*-values to be calculated. A Manhattan plot is frequently used to illustrate the GWAS results and

shows the negative logarithm of the *p*-value as a function of genomic location. SNPs with the highest significance will appear most prominently on the Manhattan plot. The *p*-value for a particular SNP in GWAS is corrected for multiple testing issues, known as Bonferroni correction. If the risk allele remains significantly more prevalent in those with the disease than in controls despite correction for multiple testing, it is said to have genome-wide significance.

In 2008, the first GWAS were published from SLE patients of European ancestry and found polymorphisms associated with lupus in the HLA region, *IRF5, STAT4, ITGAM, TNFAIP3 BLK, BANK1*, *IRF7, PXK* and *WDFY4 (*Kozyrev et al, 2008) (Hom et al, 2008) (Harley et al, 2008) (Graham et al, 2008). A GWAS of SLE patients of Han ancestry published shortly afterwards also found associations with *STAT4, BLK, IRF5,* and *TNFAIP3.* In addition, this study identified new candidate genes with associations in *IKZF1, TNFSF4, ETS1, UBE2L3,* and *RASGRP* (Han et al, 2009).

In 2009, a further GWAS of European patients with SLE discovered associations in *TNIP1, PRDM1, JAZF1, UHRF1BP1* and *IL10* (Gateva et al., 2009). In 2010, a GWAS of Chinese Han and Thai patients showed associations with polymorphisms in *ETS1* and *WDFY4* (Yang et al, 2010). A study of anti-dsDNA positive SLE patients found associations in the HLA region, *IRF5, BLK, ITGAM, STAT4, TNXB* and *TNPO3* (Chung et al., 2011). In that same year, a large replication study in a UK dataset identified a number of genes involved in type I interferon pathways with SLE associations including *IKZF1, IRF8, IFIH1* and *TYK2* (Cunninghame Graham et al, 2011).

In one of the few studies focusing on African American SLE patients; *BLK, BANK1, TNFSF4* and *CTLA4* were found to be associated (Sánchez et al, 2011). In a multi-

ancestral cohort of European, African American, Asian, Hispanic, Gullah and Amerindian SLE patients associations were found in *IRF5*, *TMEM39A* and *IKZF3-ZPBP2* (Lessard et al., 2012). Okada et al published GWAS of Japanese SLE patients with associations in *AFF1* and *LEF1* (Okada et al, 2012).

In 2012, a further GWAS of SLE patients of European ancestry again confirmed the HLA region, *IRF5, STAT4, BLK* and *TNPO3* as lupus susceptibility genes (Lee et al., 2012). A combined Chinese and Thai GWAS also confirmed association with the HLA region, *STAT4, BLK, TNFSF4* and *TNFAIP3* and described new associations in *GPR19, TET3, CD80* and *UHRF1BP1* (Yang et al, 2012). A combined GWAS of SLE and Sjögren's syndrome patients found disease associations with variants in *IRF5*, *STAT4*, *PXK*, *ITGAM*, *IRF8*, *TNFAIP3*, *BLK*, *TNPO3, JAZF1* and *TNIP1* (Martin et al., 2012).

GWAS in SLE patients have identified several potentially interesting candidate genes in biologic pathways that make sense in the context of our current knowledge of lupus pathogenesis, such as genes involved in TLR and type I interferon signalling, NFκB signalling, apoptosis and B and T cell signalling. Some of these susceptibility genes have been extensively investigated while others have yet to be examined in detail. In this thesis, we describe genotype-phenotype correlations for polymorphisms in many of these candidate genes, both on an individual level (Chapter 4) and in aggregate in a polygenic risk score (Chapter 5).

1.2.2 Monogenic lupus

Classical Complement Pathway Defects

Mutations in classical complement pathway components were the first monogenic causes of SLE to be identified and revealed valuable information about lupus pathogenesis. Mutations causing homozygous deficiencies in C1q and C4 are strongly associated with the development of SLE and will be discussed in the 'Complement' and 'Apoptosis' sections of this thesis.

Nuclease Defects

Impaired apoptosis and disposal of nuclear debris have been implicated in lupus pathogenesis and in keeping with this, nuclease defects have been identified as monogenic causes of SLE. A functional heterozygous missense mutation in *DNASE1* was previously identified in 2 unrelated Japanese SLE patients (Yasutomo et al., 2001). An autosomal recessive null mutation in *DNASE1L3* has been described in juvenile onset SLE patients from consanguineous families in Saudi Arabia (Al-Mayouf et al., 2011). These patients had a high frequency of lupus nephritis and autoantibodies including ANA, anti-dsDNA and ANCA. Dnase-1 deficient mice develop features similar to human SLE with autoantibody production and glomerulonephritis (Napirei et al., 2000).

Interferonopathies

Consistent with the pivotal role of type I interferon in the pathogenesis of SLE, a number of rare but insightful causes of monogenic lupus have been identified with aberrations in type I interferon signalling pathways, these syndromes have been coined interferonopathies. The first of these to be identified were mutations in *TREX1*, a 3'-5' DNA exonuclease with known properties as a negative regulator of interferon stimulatory cytosolic DNA (Stetson et al., 2008). TREX1 is part of the

SET protein complex in the endoplasmic reticulum that is involved in apoptosis, transcription and histone binding. Mutations in *TREX1* cause Aicardi-Goutieres syndrome (AGS), a progressive encephalopathy with cerebral calcification and raised interferon-α in the CSF (Lebon et al., 1988; Crow et al., 2006). *TREX1* mutations cause familial chilblain lupus (Rice et al., 2007). 0.5% - 2% of SLE patients carry a mutation in *TREX1* (Lee-Kirsch et al., 2007) (Namjou et al., 2011). Mutations in other genes have since been identified as the cause of AGS and interferonopathy including *RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1*, and *ADAR* (Rice et al., 2013).

An association has been described between SLE and spondyloenchondroplasia (SPENCD) (Briggs et al., 2011; Lausch et al., 2011). SPENCD is caused by mutations in *ACP5* (acid phosphatase 5, tartrate resistant) that encodes the protein tartrate resistant acid phosphatase (TRAP). Clinical features of autoimmunity seen in SPENCD include the classic hallmarks of SLE; malar rash, lupus nephritis and the presence of anti-dsDNA antibodies. Other autoimmune diseases such as Sjögren's syndrome, antiphospholipid syndrome, vitiligo, hypothyroidism and inflammatory myositis have been seen in overlap with SPENCD. A type I interferon gene expression signature and increased serum levels of type I interferon are present in SPENCD patients (Briggs et al., 2011).

Gain-of-function mutations in *IFIH1*, a cytosolic double-stranded RNA receptor gene have been shown to cause upregulation of type I interferon signalling with neurological and autoimmune features (Rice et al., 2014). *IFIH1* has previously been identified as a lupus susceptibility gene in a large scale replication study of European patients (Cunninghame-Graham et al., 2011).

A recent report has described a further interferonopathy known as SAVI (STING-associated vasculopathy with onset in infancy). Children with de novo mutations in *TMEM173* which encodes STING (stimulator of interferon genes) have neonatal onset systemic inflammation with severe cutaneous vasculitis, a pronounced type 1 interferon signature and elevated levels of interferon-induced cytokines (Liu et al., 2014). STING plays an important role in cytosolic DNA sensing and induces type I interferon gene expression via TBK1 (TANK binding kinase 1) and IRF3 (interferon response factor 3). The disease mechanisms underlying SAVI are, therefore, of interest in the context of SLE pathogenesis given the overlap with DNA-induced type I interferon production.

Defective apoptosis

Belot et al studied 3 siblings with juvenile onset lupus nephritis from a consanguineous kindred of North European ancestry and, using Whole Exome Sequencing (WES), identified a homozygous missense mutation in *PRKCD* (protein kinase Cδ) (Belot et al., 2013). This mutation leads to reduced expression and activity of its encoded protein PKC-δ. *PRKCD* is involved in deletion of autoreactive B-cells and this mutation is associated with dysregulated apoptosis and a shift to an immature B-cell phenotype. A Ca^{2+} dependent pro-apoptotic Erk signalling pathway that mediates negative selection of B-cells has recently been described. This pathway requires PKC-δ, STIM-1 (stromal interaction molecule 1) and the guanine nucleotide-exchange factor, RasGRP, to function efficiently (Limnander et al., 2011). This pathway is impaired in PKC-δ deficient mice that are known to develop B cell associated autoimmunity with expansion of immature B-cells, autoantibody production and glomerulonephritis (Miyamoto et al., 2002). Interestingly, polymorphisms in *RASGRP3* have been associated with SLE in East Asian GWAS

(Han et al., 2009). Furthermore, RASopathies, such as Noonan syndrome and Noonan-related syndromes, have been proposed as novel monogenic conditions predisposing to the development of SLE due to a growing number of case reports of co-existent RASopathy and SLE (Bader-Meunier et al., 2013).

Table 1.1: Genes involved in monogenic and syndromic lupus

Mutated Gene	**Protein Affected**	**Inheritance Pattern**	**Clinical Manifestations**
C1QA *C1QB* *CIQC*	C1q	AR	Nephritis CNS involvement Susceptibility to infection by encapsulated bacteria
C1R *C1S*	C1r/C1s	AR	Nephritis Susceptibility to infection by encapsulated bacteria
C4A *C4B*	C4	AR	Multi-systemic involvement Susceptibility to infection by encapsulated bacteria
C2	C2	AR	Musculoskeletal & skin Mild or absent renal disease Susceptibility to infection by encapsulated bacteria Pyogenic infections
C3	C3	AR	Musculoskeletal & skin Pyogenic infections
DNASE1	DNase 1	AD	SLE & Sjögren's syndrome
DNASE1L3	DNase 1L3	AR	Juvenile onset SLE Lupus nephritis
TREX1	TREX1	AD/AR	Intracerebral calcifications Psychomotor retardation Chilblain lupus
AGS5	SAMHD1	AD	Psychomotor retardation Intracerebral calcifications Chilblain lupus
ACP5	TRAP	AR	Growth retardation Spondyloenchondrodysplasia SLE & Sjögren's syndrome
PRKCD	PKCσ	AR	Musculoskeletal & serositis Lupus nephritis CNS involvement Haemolytic anaemia

1.3 Pathogenesis of SLE

1.3.1 The role of autoantibodies in SLE

Autoantibody formation is a defining feature of SLE. The detection of autoantibodies in the serum is a key factor in making the diagnosis while subsequent monitoring of autoantibody titres is used for assessing the ongoing level of disease activity and response to treatments. Development of autoantibodies precedes overt clinical manifestations of SLE by several years (Arbuckle et al., 2003).

ANA are the most frequently positive autoantibodies detected in patients with SLE, but are not specific to the disease. ANA are seen in several other autoimmune conditions including rheumatoid arthritis, Sjögren's syndrome, scleroderma, polymyositis, dermatomyositis, primary biliary cirrhosis, autoimmune hepatitis, multiple sclerosis, thyroid disease, and antiphospholipid syndrome, as well as in non-autoimmune diseases such as certain infections and malignancies. Patients with SLE who are ANA negative tend to have positive anti-Ro and /or anti-La antibodies and less renal involvement (Maddison et al., 1981).

Anti-dsDNA (double stranded DNA) antibodies are more specific and are present in approximately 70% of SLE patients and less than 0.5% of healthy individuals (Isenberg et al., 1985). Serum anti-dsDNA titres are routinely measured in clinical practice as they tend to correlate with disease activity in SLE. Higher levels usually indicate more active lupus (Swaak et al., 1979; Lloyd et al., 1981; der Borg et al., 1990; Okamura et al., 1993). Some patients, however, have discordant anti-dsDNA titres and disease activity. Some are serologically active but clinically quiescent (SACQ), while others have clinical activity with serological quiescence (CASQ) (LeBlanc et al., 1994; Gladmann et al., 2003; Steiman et al., 2012).

A matter of much debate has surrounded whether autoantibodies are a cause or consequence of SLE. The majority of studies examining the potential pathogenicity of autoantibodies in SLE have focused on the role of anti-dsDNA antibodies in lupus nephritis.

Antibody-nucleosome complexes are known to deposit in the glomerular basement membrane of the kidney and initiate complement activation leading to tissue damage (Kramers et al., 1994; van Bruggen et al., 1997; Berden et al., 1999). Studies in SLE patients and murine models of disease indicate that IgG antibodies that bind with high affinity to dsDNA are associated with more severe tissue damage than IgM or low affinity IgG antibodies (Okamura et al., 1993; Ehrenstein et al., 1995; Ravirajan et al., 1998). It has also been proposed that anti-dsDNA antibodies may have direct pathogenic effects on the kidney via cross-reactivity with renal proteins (Mostoslavsky et al., 2001).

Studies of renal biopsy specimens from lupus nephritis patients have detected several non-DNA antigens bound to IgG, including Ro, La, C1q and Sm (Mannik et al., 2003). Antibodies to C1q, a critical component in the early classical complement pathway are present in approximately 30% of SLE patients (Gunnarsson et al., 1997; Trendelenburg et al., 1999). In SLE, classical complement pathway activation is triggered by the interaction between C1q and immune complexes. Anti-C1q antibodies have been shown to be a good predictive marker of lupus nephritis flare (Siegert et al., 1993; Coremans et al., 1995; Moroni et al., 2001; Chen et al., 2012; Yin et al., 2012). The role of complement pathways in SLE will be discussed in detail later on in this thesis.

Antibodies to extractable nuclear antigens (Ro, La, Sm, and RNP) are found in up to a third of SLE patients and several lines of evidence support their role in nephritis and a severe disease phenotype. Anti-Sm and anti-RNP antibodies are more commonly seen in African SLE patients than those of European ancestry (Arnett et al., 1988; Quintero-Del-Rio et al. 2001; Cooper et al., 2002). Anti-Ro antibodies have been identified as a predictor of progression to ESRD in patients with lupus nephritis (Korbet et al., 2007). Young, African SLE patients with anti-Sm autoantibodies have a higher risk of developing nephritis (Alba et al., 2003). These RNA containing immune complexes are of relevance in the activation of TLR7 and subsequent type I interferon production. RNA containing immune complexes and their role in TLR activation will be discussed in detail in the TLR section of the introduction (Vollmer et al., 2005; Savarese et al., 2006).

1.3.2 Complement pathways in SLE

The role of complement pathways in SLE is complex as complement may both prevent and exacerbate disease. Inherited deficiencies of the classical complement pathway increase susceptibility to SLE. Complement is implicated at the effector phase of SLE when complement is known to deposit in inflamed tissue. Early components of the classical pathway, particularly C1q have important functions in the clearance of apoptotic cells, removing a potent source of antigenic material (Botto et al., 1998). Complement also plays a role in T and B cell activation thereby maintaining immune tolerance (Kemper et al., 2003).

There are three distinct pathways within the complement system, the classical complement pathway, the alternative complement pathway and the lectin pathway, linking innate and adaptive immunity. Antibody-antigen complexes initiate

activation of the classical complement pathway involving the proteins, C1q, C1s, C1r, C2, C4 and C3. All three complement pathways merge at C3 which is then converted to C3a and C3b. C3b then forms part of C5 convertase which cleaves C5 into C5a and C5b. C5b, C6, C7, C8 and C9 together form the membrane attack complex (MAC) that binds to cell surfaces and initiates lysis.

Complement activation and consumption is common in SLE patients and results in hypocomplementaemia. C3 and C4 serum levels are routinely used in clinical practice to monitor disease activity with low C3/C4 levels, or a temporal reduction in consecutive serum levels being suggestive of active disease. Deposition of complement is evident at sites of inflammation in SLE, particularly in the kidney and skin. Deposition of complement and immunoglobulins at the dermal-epidermal junction, known as the 'lupus-band' test has been used as a diagnostic test in SLE.

Mutations causing homozygous deficiencies of classical complement pathway components are strongly associated with the development of SLE. C1q deficiency whilst rare with <100 cases reported in the literature, is highly penetrant and the strongest known genetic risk factor for the development of SLE. Patients with C1q deficiency tend to be of juvenile onset and have a severe clinical phenotype.

Other known mutations of the classical complement pathway include mutations in *C2, C3* and *C4*. C2 deficiency results in a clinical phenotype of SLE, increased susceptibility to infection, anti-C1q antibodies and anticardiolipin antibodies (Jonsson et al., 2007). *C4* has 2 isoforms, *C4A* and *C4B*. Complete C4 deficiency is rare. Low CNV (copy number variation) of *C4A* has been associated with SLE while increased CNV is protective (Yang et al., 2007). Homozygous C3 deficiency is rare

and those with C3 deficiency have a much lower rate of SLE development than those who are C1q or C4 deficient (Botto et al., 1993).

Encouraging results from murine models of nephritis have led to the targeting of complement in the treatment of lupus nephritis with a monoclonal antibody against C5 (eculizumab) (Wang et al., 1996). Successful blockade of complement has been clinically efficacious in haemolytic uraemic syndrome and dense deposit disease (Vivarelli et al., 2012; Wong et al., 2013). An early phase trial of 24 SLE patients with low disease activity showed good safety and tolerability. The prohibitively high cost of the drug has limited the progression of further clinical trials, however (Barilla-Labarca et al., 2013).

1.3.3 Apoptosis in SLE

Apoptosis is a genetically controlled process whereby specific endonucleases cleave DNA and generate nucleosomes, resulting in death of the cell followed by disposal of its contents (Hengartner et al., 2000; Savill et al., 2000). Chromatin condensation, fragmentation of DNA, membrane blebbing and externalisation of phosphatidyl serine (PS) are cardinal features of apoptosis. If apoptotic cells are not disposed of properly, they become secondary necrotic cells, triggering the release of danger signals and ultimately resulting in loss of tolerance and development of autoimmunity. Nucleosomes produced during apoptosis are a major autoantigen source in SLE (Mohan et al., 1993; Amoura et al., 1997; Bruns et al., 2000). Nucleosomes can bind antibody forming immune complexes and then deposit in tissue (Berden et al., 1999; Licht et al., 2001). Lupus autoantigens are known to be located on the surface of apoptotic cells and on surface blebs (Casciola-Rosen et al., 1994, 1995).

Apoptosis is dysregulated in SLE in two ways, firstly there is an increased rate of apoptosis and secondly a decreased clearance of apoptotic bodies by phagocytic cells. Increased apoptosis has been demonstrated in lymphocytes, neutrophils and monocytes from patients with SLE (Emlen, et al., 1994; Courtney et al., 1999; Shoshan et al., 2001; Kaplan et al., 2002; Ren et al., 2003). This increased rate of apoptosis in phagocytic cells increases the production of apoptotic debris and impairs clearance of this material by the cells themselves. T lymphocytes from patients with SLE demonstrate increased expression of the apoptotic ligands TWEAK, TRAIL and FasL (Kaplan et al., 2002). Increased apoptosis has also been seen in circulating endothelial cells in patients with SLE (Rajogopalan et al., 2004).

Decreased clearance of apoptotic material by phagocytic cells has been demonstrated in patients with SLE (Hermann et al., 1998). In support of the role of complement in apoptosis, there is impaired uptake of apoptotic cells by macrophages in the presence of serum depleted of C1q and C3 (Mevorach et al., 1998; Taylor et al., 2000). Macrophages from SLE patients have defective ability to internalise apoptotic cells even in the presence of normal serum (Tas et al., 2006). Genetic mutations in SLE patients support the concept of reduced clearance of apoptotic debris in lupus pathogenesis, including mutations in *C1Q* and *DNASE1*. C1q deficient mice develop autoantibodies, glomerulonephritis and large quantities of glomerular apoptotic bodies (Botto et al., 1998). As discussed earlier in this thesis, patients with homozygous *C1Q* mutations have a high risk of developing SLE. Mutations in *DNASE1* and *DNASE1L3* have been identified as monogenic causes of SLE (Yasutomo et al., 2001; Al-Mayouf et al., 2011). Dnase-1 deficient mice develop features similar to human SLE with autoantibody production and glomerulonephritis

(Napirei et al., 2000). Furthermore C1q and DnaseI have been shown to cooperate in degrading chromatin (Gaipl et al., 2004).

A recently described monogenic cause of SLE arising from a mutation in *PRKCD* (protein kinase Cδ) may primarily exert its effect through apoptosis. *PRKCD* is involved in the deletion of autoreactive B cells. Patients with this mutation had evidence of dysregulated apoptosis and a shift to an immature B-cell phenotype (Belot et al., 2013).

1.3.4 Toll-like receptors in SLE

Toll-like receptors (TLRs) are a family of innate immune pattern-recognition receptors (PRRs) that recognise 'danger signals' or pathogen-associated molecular patterns (PAMPs) including viral nucleic acids. Activation of TLRs sets in motion a complex system of downstream adaptor proteins and signalling pathways. When TLRs sense endogenous RNA and DNA in immune complexes, type I interferon is induced with resultant autoimmunity (Akira et al., 2001; Schnare et al., 2001).

As opposed to other TLRs on the cell surface, TLR3, TLR7/8 and TLR9 are endosomal and thus have restricted access to foreign nucleic acids. TLR7 senses ssRNA and therefore can be activated by SLE immune complexes containing RNA (Leadbetter et al., 2002; Lau et al., 2005). The lupus autoantigen U1snRNP RNA has been identified as an endogenous ligand for TLR7 and 8 (Vollmer et al., 2005; Savarese et al., 2006). The BXSB-*Yaa* (*Y-linked autoimmune acceleration*) lupus-prone mouse model is the consequence of a translocation from the telomeric end of the X chromosome onto the Y chromosome. This results in the duplication of a number of genes including the gene encoding TLR7, which is deemed to be the major functional contributor to this phenotype (Pisitkun et al., 2006; Subramanian et

al., 2006). These mice spontaneous develop SLE like features and males have disease onset at a younger age than females. TLR9 senses unmethylated CpG DNA and is activated by DNA containing immune complexes (Boule et al., 2004; Means et al., 2005). Plasmacytoid dendritic cells (pDCs) which have been identified as the primary source of type I interferon production in humans are known to constitutively express TLR7 and TLR9 (Ronnblom et al., 1983).

Polymorphisms in *TLR7* have been associated with SLE in an East Asian candidate gene study. Males were found to have a stronger association than females and an enhanced type I interferon gene expression signature (Shen et al., 2007). Subsequent studies found an association with other *TLR7* variants and SLE but did not replicate the link with male disease (Deng et al., 2010; Kawasaki et al., 2011). Increased *TLR7* CNV is a risk factor for juvenile onset SLE (Garcia-Ortiz et al., 2010). *TLR9* polymorphisms have not been associated with SLE with genome wide significance, as yet. A number of studies have shown weak associations of *TLR9* variants with SLE (Tao et al., 2007; Xu et al., 2009; dos Santos et al., 2012). *TLR9* is upregulated in B lymphocytes of SLE patients (Papadimitraki et al., 2006). Other studies, however, have not shown an association between *TLR9* polymorphisms and SLE (Ng et al., 2005). While studies of *TLR9* in SLE appear contradictory, the overall consensus on the available evidence is that *TLR9* contributes to the development of SLE rather than having a protective role.

In addition to *TLR7* and *TLR9* polymorphisms, variants in genes involved in signalling downstream of TLRs have been associated with SLE. The most strongly associated and extensively investigated candidate is *IRF5* (Harley et al, 2008; Hom et al, 2008; Han et al. 2009; Lee et al., 2012). Polymorphisms in *IRF7* and *IRF8* have

also been associated with SLE with genome wide significance (Harley et al., 2008; Cunninghame-Graham et al., 2011; Li et al., 2011).

TLR7 and TLR9 represent interesting therapeutic target in SLE. A dual TLR7/TLR9 inhibitor (IRS954) reduced autoantibody production, proteinuria and histologic severity of glomerulonephritis in (NZB NZW) F1 mice (Barratt et al., 2007). Definitive clinical trials in patients with SLE are pending.

1.3.5 Type I interferon in SLE

Type I interferon family members play a major role in innate immunity and host viral defense. The type I interferons present in humans are IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. Interferon-α is a homogeneous family of proteins encoded by 13 different genes. During viral infection, viral nucleic acids bind endosomal TLRs (TLR3, TLR7, TLR8, TLR9), leading to the activation of intracellular signaling pathways. Type I interferon production ensues, that in turn activates the expression of interferon-inducible genes and, ultimately results in viral elimination.

Several lines of evidence link type I interferon to the pathogenesis of lupus. It is well established that patients with SLE have high serum levels of interferon-α (Hooks et al., 1979; Ytterberg et al., 1982). It has also been noted that viral hepatitis and oncology patients receiving interferon-α therapy develop autoimmune side-effects. An estimated 20% of patients receiving interferon-α therapy develop a positive ANA detectable in their blood or other autoimmune features while 1% develop clinical SLE (Ronnblom et al., 1990; Ronnblom et al., 1991; Kalkner et al., 1998). Serum interferon-α levels have been shown to correlate with disease activity and severity in SLE (Bankhurst et al., 1987; Bengtsson et al., 2000).

Debris from apoptotic cells has been shown to induce interferon-α production *in vitro* (Vallin et al., 1999, Bave et al., 2000, 2001). Apoptotic and necrotic U937 cells (a myeloid leukaemia cell line) release material that, when combined with SLE immunoglobulin, induce interferon-α production (Lövgren et al., 2004). As discussed, above, SLE patients have increased levels of apoptosis and decreased clearance of apoptotic debris. This provides an endogenous source of chromatin that, leads to autoantibody production and immune complex formation. Serum from patients with SLE can induce monocytes to differentiate into DCs in an interferon-α dependent process (Blanco et al., 2001). Both DNA and RNA-containing immune complexes can induce interferon-α production (Lövgren et al., 2004, 2006). More recently, neutrophil extracellular traps (NETs) have been identified as major producers of type I interferon, triggering pDC activation via TLR9 (Garcia-Romo et al., 2011; Lande et al., 2011; Villaneuva et al., 2011).

While not all SLE patients have raised serum interferon-α, the majority have an interferon gene expression signature in PBMCs particularly in the early stages of their disease (Baechler et al., 2003; Bennett et al., 2003). Gene expression microarray profiles demonstrate upregulation of interferon-inducible genes, particularly in patients with severe SLE manifestations such as lupus nephritis and CNS disease. Haematologic findings such as haemolytic anaemia, leucopenia, lymphopenia and thrombocytopenia are associated with a somewhat less pronounced interferon signature. Milder disease features such as serositis and photosensitivity do not significantly correlate with the interferon signature (Bennett et al., 2003). In paediatric-onset SLE, which is generally deemed to have a more severe clinical phenotype than adult disease, a pronounced interferon-α signature is seen and has

been shown to down-regulate after high dose intravenous corticosteroid therapy (Bennett et al., 2003).

Additional evidence of the contribution of type I interferon to lupus pathogenesis is provided by the regularity with which variants in genes involved in interferon signalling pathways are identified in SLE GWAS including *IRF5, IRF7, IRF8, STAT4, TYK2,* and *IFIH1*. Furthermore, there is a strong link between monogenic causes of lupus and interferonopathies. Aicardi-Goutieres syndrome (AGS), the prototypic interferonopathy, characterised by raised CSF interferon-α levels, cerebral calcification and progressive encephalopathy is a prime example of this (Lebon et al., 1988). The first genetic mutation underlying AGS was identified in *TREX-1,* a DNA exonuclease that is also associated with SLE and has been discussed in detail in the 'Monogenic Lupus' section of this thesis (Lee-Kirsch et al., 2007). Since that discovery a number of other genes have been implicated in AGS and interferonopathies including *RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1,* and *ADAR* (Rice et al., 2013). More recently, gain-of-function mutations in *IFIH1,* a cytosolic double-stranded RNA receptor gene have been shown to cause a spectrum of disease phenotypes associated with upregulation of type I interferon signalling (Rice et al., 2014). *IFIH1* has previously been identified as a lupus susceptibility gene in a large scale replication study of European patients (Cunninghame-Graham et al., 2011).

A number of therapeutic strategies are under development to target interferon-α in clinical practice, including the use of monoclonal antibodies directed both against interferon-α itself and against the type I interferon-α receptor and immunization against interferon-α. Monoclonal antibodies targeting interferon-α, currently in clinical development include sifalimumab and rontalizumab.

Sifalimumab has been tested in a phase I clinical trial and was found to be safe and well tolerated with no increased rate of viral infections. Approximately 60% of the patients recruited had a positive interferon signature at study onset and a dose-dependent reduction in the expression of interferon-inducible genes was evident in these individuals (Merrill et al., 2011). A phase II study, showed similar inhibition of the interferon signature but no differences in clinical response were seen between the sifalimumab and placebo groups (Merrill et al., 2011). The other monoclonal antibody in development against interferon-α, rontalizumab, has produced a similar reduction in the expression of interferon-inducible genes in clinical trials and there were some indications that patients treated with rontalizumab fared better than those who received placebo (McBride et al., 2012; Kalunian et al., 2012).

1.3.6 The role of monocytes in the pathogenesis of lupus nephritis

Over the past decade there has been an accumulation of evidence from studies involving patients with proliferative lupus nephritis and from experiments in lupus mouse models suggesting an important role of monocytes in renal damage. Monocytes are a heterogenous population of blood leucocytes that play important roles in the innate immune system. In mice, there are two phenotypically and functionally different subsets, $Gr1^-$ and $Gr1^+$. A subset of $Gr1^-$ 'resident' monocytes patrol the endothelium of blood vessels, allowing rapid tissue invasion by these cells in the case of local tissue damage or infection (Auffray et al., 2007).

Selective recruitment of $Gr1^-$ monocytes in lupus mouse models of nephritis

Expansion and activation of $Gr1^-$ patrolling monocytes has been demonstrated in several lupus mouse models of nephritis. The onset of proliferative nephritis and proteinuria in NZB/NZW F_1 mice is associated with the expression of chemokines

such as CCL2 (MCP-1), CCL3 (MIP-1α), and CCL5 (RANTES) that mediate glomerular infiltration by myeloid cells (Schiffer et al., 2008). A large population of $CD11b^{+}$ $Gr1^{-}$ monocytes are present in the kidneys and peripheral blood of NZB/NZW F_1 mice during active renal disease. Further histologic analysis confirms the disappearance of these cells early in remission. It remains to be determined whether these cells die in situ following immunosuppressive therapy or migrate out of the kidney (Schiffer et al., 2008).

MRL/*lpr* mice spontaneously develop autoantibodies, circulating immune complexes and progressive nephritis resembling human proliferative glomerulonephritis. By 20 weeks these mice develop progressive renal damage, including hypercellular glomeruli, crescent formation, glomerulosclerosis, and prominent interstitial mononuclear cell infiltrates (Inoue et al., 2005).

A unique cellular feature in the BXSB -*Yaa* lupus mouse model, is the striking expansion of monocytes. By 8 months of age, monocytes account for 50% of peripheral blood mononuclear cells in BXSB -*Yaa* mice (Santiago-Raber et al., 2009). This monocytosis is characterized by selective expansion of the $Gr1^{-}$ subset (Amano et al., 2005).

$CD16^{+}$ monocytes in lupus nephritis patients, clinical significance and hallmark of disease activity

There are three subsets of monocytes in humans distinguished from each other based on their phenotype and cytokine production ($CD14^{+}$ $CD16^{-}$, $CD14^{+}CD16^{+}$and $CD14^{dim}CD16^{+}$). Interestingly, the human subset orthologous to murine patrolling $Gr1^{-}$ monocytes is characterized by expression of $CD16^{+}$ (FcgRIII). Mean glomerular $CD16^{+}$ monocytes counts are higher in class IV proliferative nephritis than in other

nephritis classes and positively correlate with blood urea nitrogen levels, anti-DNA antibody titres, and proteinuria. Glomerular $CD16^+$ monocyte counts negatively correlate with serum albumin levels, complement levels and estimated glomerular filtration rate (Yoshimoto et al., 2007). Glomerular $CD16^+$ monocyte counts correlate with histopathological activity index but not with chronicity index (Yoshimoto et al., 2007).

Glomerular monocytes have been reported to be more prevalent on immunostaining of class IV-G nephritis renal biopsies than in class IV-S (Hill et al., 2005). The presence of glomerular monocytes on repeat renal biopsy six months after induction of immunosuppressive therapy is negatively associated with renal survival, with survival rates of 35.5% at 10-years in those with persistence of monocytes on biopsy versus 75.8% survival for those without (Hill et al., 2001).

$CD16^+$ monocytes are present in the urine of patients with proliferative forms of nephritis including rapidly progressive crescentic glomerulonephritis, membranoproliferative glomerulonephritis and lupus nephritis unlike patients with non-proliferative renal disease such hereditary nephropathy and renal calculi. Increased urinary CD16+ monocytes are observed during acute exacerbations of urinary abnormalities and their disappearance closely precedes improvements in urinary abnormalities in patients with proliferative glomerulonephritis and therefore could potentially serve as a useful biomarker of glomerular disease activity (Hotta et al., 1999).

The role of Fc gamma receptors and myeloid cells in lupus nephritis

Extracellular nucleic acids do not normally trigger cell activation in part because receptors for nucleic acids such as TLRs 3, 7, 8 and 9 are located in endosomes. However, when part of an immune complex, nucleic acids can be internalised into endosomes by Fc receptor bearing cells, and potently trigger TLR mediated activation of immune cells, which result in the production of type I interferon (Lövgren et al., 2004) . This is due to the fact that myeloid cells can internalise immune complexes via Fcγ receptors. NZB/NZW F1 lupus prone mice bearing a deletion of the common Fcγ chain do not develop proteinuria or histological evidence of lupus nephritis despite producing anti-DNA antibodies, circulating immune complexes and IgG and C3 deposits in glomeruli (Clynes et al., 1998). Therefore, antibody deposition alone is insufficient for the development of lupus nephritis; activation of FcR-bearing myeloid cells such as monocytes/ macrophages via immune complexes is required for pathogenicity to develop (Bergtold et al., 2006). Indeed, $Gr1^-$ monocyte expansion in BXSB *Yaa* mice is characterized by high expression of activating FcγRIV and low expression of inhibitory FcRγIIB (Santiago-Raber et al., 2009).

The role of monocytes and TLR pathway activation in lupus nephritis

Immunostaining of renal sections in MRL-*lpr* lupus-prone mice shows TLR7 expression in infiltrating macrophages and repeated injection of the TLR7 ligand imiquimod aggravates glomerular disease in these mice with evidence of increased glomerular immune complex deposition and expression of CCL2 (Pawar et al., 2006). Inhibition of TLR-7 signaling, results in reduced serum levels of autoantibodies including anti-Sm, improved activity and chronicity indices and

significantly decreases renal glomerular and interstitial macrophage infiltrates in MRL/ *lpr* mice (Pawar et al., 2007).

TLR7-dependent nucleic acid 'danger' signals trigger intravascular retention of patrolling monocytes by the endothelium. Following this, monocytes recruit neutrophils in a TLR7-dependent manner to mediate focal necrosis of endothelial cells, with monocytes then removing the ensuing cellular debris (Carlin et al., 2013).

F*ractalkine antagonists ameliorate glomerular damage in lupus mouse models*

Fractalkine (CX3CL1) and its receptor, CXRCR1, are known to mediate cell migration and adhesion (Imai et al., 1997) (Fong et al., 1998). Fractalkine is expressed on endothelial cells and is a membrane-bound chemokine that attracts cells expressing its receptor, CX3CR1, including $CD16^+$ monocytes (Ancuta et al., 2003). Renal histologic sections from lupus nephritis patients demonstrate fractalkine expression in the mesangial area and along the capillary wall in proliferative nephritis classes III and IV. There is a strong correlation between glomerular fractalkine expression, $CD16^+$ monocyte count and histopathological activity index (Yoshimoto et al., 2007).

MRL/*lpr* mice show a progressive increase in renal fractalkine expression from 12 to 20 weeks predominantly in glomerular endothelial cells and to a lesser degree in mesangial cells (Inoue et al., 2005). Antagonism of fractalkine by subcutaneously injecting MRL/*lpr* mice with an N-terminal truncated fractalkine analog transfected into a non-metastatic fibroblast cell line delays onset and slows progression of nephritis. Inhibition of fractalkine resulted in significantly lower levels of interstitial mononuclear cell infiltration, glomerular hypercellularity, crescent formation and glomerulosclerosis as compared to control MRL/*lpr* mice. There was a 75%

reduction in intra-glomerular macrophages in fractalkine antagonist treated mice compared to control, although no significant difference in intra-glomerular T cells was seen (Inoue et al., 2005). Proteinuria was significantly reduced in mice who received the fractalkine antagonist. Other features of autoimmunity such as pneumonitis, lymphadenopathy and splenomegaly did not improve with fractalkine inhibition in the MRL/*lpr* model. Circulating immune complexes and anti-DNA antibody levels were not reduced by fractalkine antagonism (Inoue et al., 2005).

Further studies have demonstrated elevated fractalkine expression and $CD16^+$ monocyte accumulation within proliferative glomerular lesions in SCID mice, which lack T and B cells. Antagonism of fractalkine in this model protected against the development of glomerular endocapillary proliferative lesions and significantly reduced glomerular $CD16^+$ monocyte counts (Nakatani et al., 2010). It appears that, by stimulating $CD16^+$ monocyte accumulation, fractalkine contributes to the development of endocapillary proliferative glomerular lesions and thus represents a potentially important therapeutic target in ameliorating glomerular injury in lupus nephritis.

These data strongly suggest that monocytes play an important role in the pathogenesis of lupus nephritis. Monocytes may represent a potential biomarker of disease activity with the extent of their presence being a predictor of poor renal outcome. Monocytes and the pathways governing their recruitment to the kidney and activation may represent legitimate therapeutic targets in the treatment of proliferative lupus nephritis.

1.4 Thesis overview

1.4.1 Thesis outline

Chapter 1 has provided an introduction to SLE. Important concepts in pathogenesis have been discussed including the role of autoantibodies, TLRs, dysregulated apoptosis, complement pathways and type I interferon. The genetics of SLE has been introduced with an overview of lupus susceptibility genes identified in GWAS and monogenic causes of SLE that provide enlightening insights into lupus pathogenesis.

Chapter 2 is a comprehensive clinical characterisation of the 164 lupus nephritis patients who were recruited for this study with the aim of identifying patients with a severe clinical phenotype who might represent good candidates for whole exome sequencing (WES). As an extension of this, Chapter 3 focuses on histopathological findings in our lupus nephritis cohort specifically ISN/RPS class of nephritis and CD68 glomerular immunostaining.

Chapter 4 provides an in-depth description of genotype-phenotype relations of polymorphisms in lupus susceptibility genes identified in GWAS with clinical parameters in lupus nephritis patients. This is an attempt to try to make sense of GWAS findings in everyday clinical practice. In Chapter 5, the cumulative effect of these polymorphisms is analysed by compiling a polygenic risk score.

Chapter 6 introduces WES and discusses sequencing results of 8 families with clustering of lupus nephritis in first degree relatives. Adopting this approach has culminated in a shortlist of novel candidate genes under consideration for future functional testing.

Chapter 7 summarizes the main findings of this research and discusses our data in the context of what is already known in the literature. Limitations of this research are discussed as well as an overview of recommendations and future directions.

1.4.2 Thesis aims

This thesis aims to explore genotype-phenotype relations in lupus nephritis. The first aim is to constitute and characterize a cohort of lupus nephritis patients; clinically, including renal outcomes and familial history, genetically, including susceptibility polymorphisms identified in GWAS and potential novel causative genes by exome sequencing of selected patients, serologically including autoantibody profile and histologically as per the ISN/RPS classification in conjunction with investigation of monocyte/ macrophage enrichment on renal biopsy.

The second aim is to integrate these clinical, genetic, serological and histologic findings in different clinical forms of lupus nephritis with a view to testing if polymorphisms in lupus susceptibility genes correlate with clinical data, histopathology findings or autoantibody profile.

Our final aim, adopting a Mendelian approach by exome sequencing familial lupus nephritis is to identify novel causative genetic mutations with a view to future functional validation, improving our understanding of lupus pathophysiology.

Chapter 2

Clinical characterisation of lupus nephritis cohort

The purpose of this chapter is to:

1. Provide an overall clinical and demographic description of the cohort of lupus nephritis patients recruited for this study.
2. Identify patients with a severe clinical phenotype of lupus nephritis.
3. Determine if gender, ancestry, juvenile onset disease or family history of SLE associate with severe disease.

2.1 Ethical Approval and Recruitment Centres

Ethical approval for this research was given in March 2010 by the Outer South East London Research Ethics Committee (REC reference number 10/H0805/3) for the project entitled 'CD14-CD16+ peripheral blood monocytes as both a reliable biomarker and a potential therapeutic target in lupus nephritis.' This ethical approval was extended to facilitate patient recruitment from University College London Hospitals under the auspices of UCL/UCLH/RF Joint Biomedical Research Unit.

When this initial ethical approval expired a second application was submitted entitled 'Investigation of immune regulation and cellular response in autoimmune and inflammatory rheumatic diseases' and a favourable opinion was given in September 2011 by the London City Road and Hampstead Research Ethics Committee (REC reference number 11/LO/1433). For both sets of ethical approval, the study sponsor was Guy's and St Thomas' NHS Foundation Trust Research and Development Department.

Patients were recruited from the Louise Coote Lupus Unit at St Thomas' Hospital (Professor David D'Cruz) (Dr Yousuf Karim), the Nephrology Department, Guy's Hospital (Dr Mike Robson) and the Rheumatology Department at UCLH (Professor David Isenberg). In addition, a family with clustering of lupus nephritis was recruited from the Rheumatology Department at Lahore, Pakistan (Dr Saira Khan and Dr Dr Nighat Ahmad). Ethical approval for enrolling this family in the study was obtained locally.

2.2 Study Participants

164 biopsy-proven lupus nephritis patients were recruited from the designated clinical centres from July 2010 to January 2012. Serum, whole blood for DNA extraction and PBMCs (peripheral blood mononuclear cells) were stored for each patient recruited. Paraffin-embedded renal biopsy slides were obtained where possible. In families with clustering of lupus nephritis, unaffected family members were invited to participate. 11 unaffected family members were recruited in total.

Inclusion criteria included patients with a definite diagnosis of SLE according to the American College of Rheumatology (ACR) revised classification criteria (Hochberg et al., 1997), patients with biopsy-proven lupus nephritis, and patients capable to giving written informed consent prior to enrolment in the study. Individuals who were pregnant or breast-feeding and those unable to give written informed consent were excluded from the study.

2.3 Clinical Variables

Clinical variables explored in this study included gender, age at diagnosis of nephritis, family history of SLE, and ancestral background. Autoantibody positivity was ascertained including ANA, anti-dsDNA, anti-Ro, anti-Sm and anti-RNP. Long-term renal outcomes in lupus nephritis patients were assessed using the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) scoring system (Collins et al., 2002). Information regarding a concurrent diagnosis of Antiphospholipid syndrome (APS) and/or the presence of anti-cardiolipin antibodies (aCL) and lupus anticoagulant (LA) was also gathered. Those with APS met the Sapporo classification criteria (Lockshin et al., 2000).

2.4 Statistical Analysis

Continuous variables were described as mean with standard deviation (SD). Categorical variables were compared using Pearson's Chi-squared ($\chi 2$) test, except in instances where counts were <5, when a Fisher's exact test was used. The D'Agostino-Pearson omnibus normality test was used to ascertain if data was parametrically or non-parametrically distributed. Student's t-test or Wilcoxon rank sum tests were used to compare continuous variables that were normally or non-normally distributed, respectively. Correlation between two quantitative variables was assessed using Pearson's correlation coefficient, r, giving a between +1 and −1, where 1 is a total positive correlation, 0 is no correlation, and −1 is a total negative correlation. A value of $p<0.05$ was considered to be significant. Analysis was carried out using GraphPad Prism 6 software.

2.5 Materials and Methods

2.5.1 Autoantibody testing

Autoantibody profiles were performed in all patients at either Guy's and St Thomas NHS Foundation Trust or University College London Hospital including ANA (anti-nuclear antibodies), anti-double stranded DNA, anti-Ro (SS-A), anti-RNP (ribonucleoproteins) and anti-Sm (Smith antigen) antibodies using a standardized counterimmunoelectrophoresis (CIE). Availability of autoantibody results were as follows; ANA 99% (n=162), anti-dsDNA 98% (n=160), anti-Ro 93% (n=152) and 92% (n=151) for anti-RNP and anti-Sm. Screening for LA was performed using the dRVVT (dilute Russell's viper venom time) assay in accordance with standard methods (Miyakis et al., 2006). Confirmatory tests used a combination of mixing studies and correction with phospholipid. IgG and IgM aCL antibodies were

measured using a standardized ELISA (Harris et al., 1986). The normal reference range is 0-7 U/ml and levels above 20 U/ml (3 SD above the mean of the normal range) were considered moderate to high titres. Anti-C1q and anti-beta-2-glycoprotein-1 (anti-β2-GP1) antibodies were available for a minority of patients only and hence were not included.

2.5.2 NKF KDOQI scoring of chronic kidney disease

The degree of chronic kidney disease was assessed as per the NKF KDOQI scoring system as follows; Stage 1, kidney damage with normal or elevated glomerular filtration rate (GFR), Stage 2, kidney damage with mild reduction in GFR (60-89 mL/min); Stage 3, moderate reduction in GFR (30-59 mL/min); Stage 4, severe reduction in GFR (15-29 mL/min); Stage 5, kidney failure (GFR<15 or dialysis). Kidney damage was defined as 'pathologic abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies' as per NKF KDOQI (Clinical Practice Guidelines for Chronic Kidney Disease: evaluation, classification and stratification. 2002). Patients with KDOQI stages 4 and 5 were classified as having advanced renal impairment in our lupus nephritis cohort.

2.6 Results

2.6.1 Overall description of lupus nephritis cohort

The study cohort was comprised of 85% (n=139) females and 15% (n=25) males. The mean current age of the cohort at study enrolment was 38.3 years ± 11.4 years. The overall mean age at diagnosis of nephritis was 25.8 ± 11.2 years. The mean disease duration in the group is 11.8 ± 7.5 years. 27% (n=44) of the study group were of juvenile onset as defined by diagnosis of nephritis before 18 years of age.

19% (n=31) were diagnosed with lupus nephritis before the age of 16 years and 2% (n=4) under the age of 10 years.

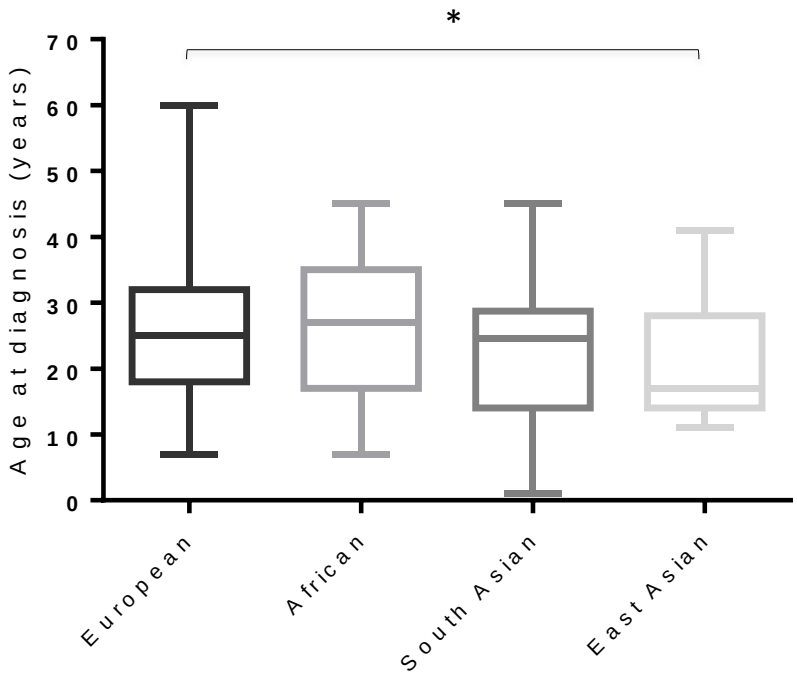
2.6.2 A clinical comparison of lupus nephritis in patients of different ancestral backgrounds

41% (n=68) of the cohort were of European origin, predominantly from the United Kingdom. 33% (n=54) were of African ancestry, the majority being of Afro-Caribbean descent and others from Nigeria, Ghana, Sierra Leone and Uganda. 13% (n=21) were of South Asian extraction, all from India and Pakistan. 9% (n=14) were East Asian, from China, Vietnam and Singapore. 4% (n=7) were classified as outliers from the 4 main ancestral groups, 2 of these were from the Middle East and 5 reported parental admixture. Of these 3 had an African parent and a European parent, 1 had an African parent and an East Asian parent and 1 had a European parent and an East Asian parent. Population stratification will be discussed in more detail in the section pertaining to ImmunoChip analysis.

Females accounted for 87% (n=47) of the African patients, 85% (n=58) of Europeans, 71% (n=15) of South Asians and 86% (n=12) of those of East Asian extraction. Juvenile onset disease was seen in equal measure in African and European patients at 24% each. The youngest onset patients were of East Asian ancestry with 50% diagnosed before age 18 years (Figure 2.1). Confirmed first degree family history of lupus nephritis was seen in 20% (n=11) of African patients and 14% of South and East Asian patients (n=3, n=2 respectively). None of the European patients had a first degree relative with lupus nephritis. East Asian patients were most likely to progress to ESRD at 14% (n=2), followed by African patients at 11% (n=6), South Asian patients at 10% (n=2) and European patients at 6% (n=4).

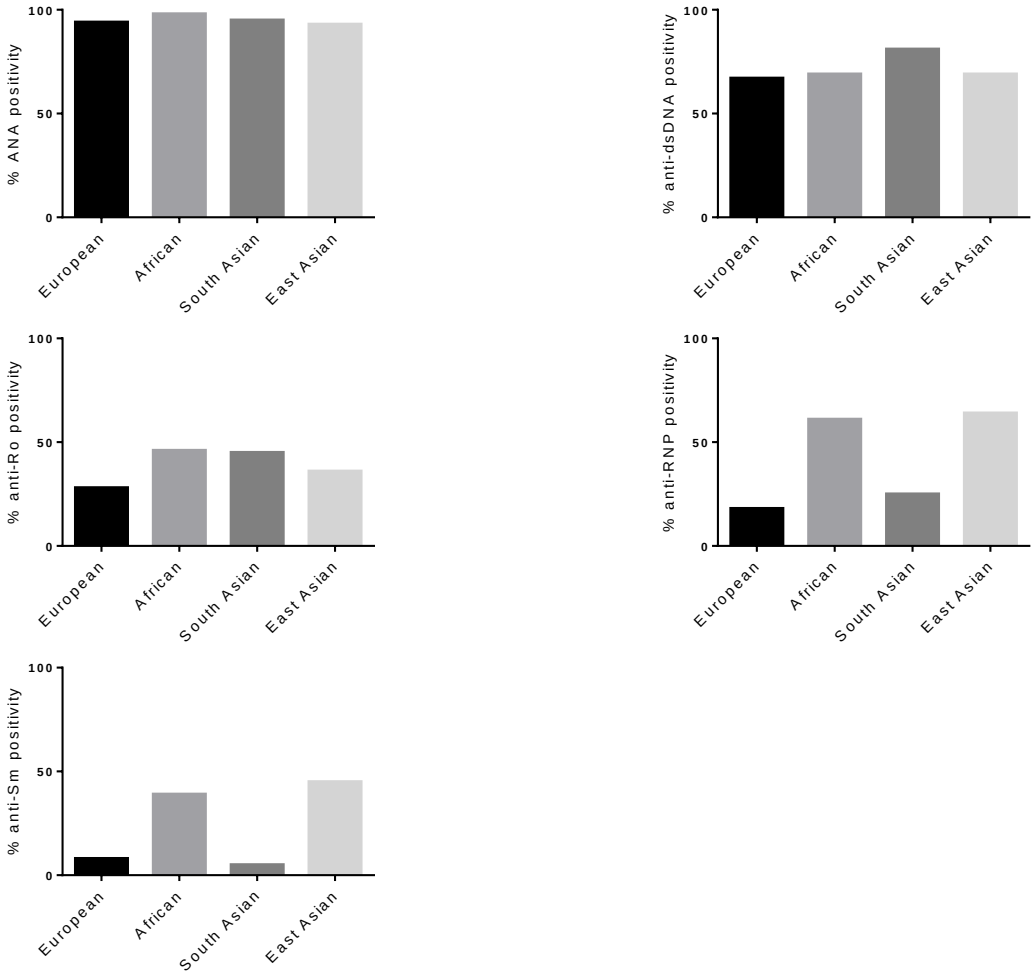
The distribution of ISN/RPS classes in different ancestral background in lupus nephritis is available in Appendix 1.

Figure 2.1: Age at diagnosis of lupus nephritis as per ancestry



Mean age at diagnosis in lupus nephritis patients of European ancestry was 27.1 ±11.9 years, Africans 26.8 ±10.7 years, South Asians 23.9 ±12.0 years and East Asians 20.9 ±9.1 years. East Asian patients were significantly younger at diagnosis of nephritis than European patients (p=0.042), otherwise there were no statistical differences between the groups.

Figure 2.2: Autoantibody positivity as per patient ancestry



ANA positivity was 98%, 94%, 95% and 93% for European, African, South Asian and East Asian respectively. Anti-dsDNA was highest in South Asian individuals at 81%. European and East Asian patients both had 69% anti-dsDNA positivity and 67% in Africans. Anti-Ro antibodies were most frequent in African and South Asian nephritis patients at 46% positivity. 28% of Europeans and 36% of East Asian were anti-Ro positive. Anti-RNP antibodies were seen in 61% of African and 64% of East Asians patients. European and South Asian individuals had less frequent anti-RNP antibodies at 18% and 25% respectively. Similarly anti-Sm antibodies were highest in African and East Asian patients at 39% and 45% positivity. South Asians and Europeans were less likely to have anti-Sm antibodies at 8% and 5% respectively.

2.6.3 Familial lupus nephritis

During clinical assessment 13% (n=22) of patients randomly recruited self-reported a family history of SLE. 4 of these relatives with biopsy proven lupus nephritis were subsequently recruited to the study resulting in a total of 26 patients with a family history of SLE in our cohort. A first-degree family history was defined as having parents, siblings or children affected with SLE. A second-degree family history was defined as having affected grandparents, grandchildren, aunts, uncles, nephews, nieces or half-siblings. A third-degree family history was defined as having affected first-cousins, great-grandparents or great grandchildren. 10% (n=16) self-reported a first degree family history which included sibling pairs and parent-child combinations. 3% (n=5) reported a second degree family history and 3% (n=5) a third degree family history. All self-reported first degree family relatives were confirmed with the exception of one. Relatives with biopsy proven lupus nephritis were also recruited to the study where possible. In addition, unaffected family members were recruited when available to facilitate WES studies.

Table 2.1: Lupus nephritis patients with a self-reported first degree family history of SLE

Subject Identifier	Gender	Ancestry	Juvenile Onset	Clinical Details of Relative
SLE 19	F	African	No	Brother with confirmed nephritis Sister with non-renal SLE
SLE 24	F	African	Yes	Brother with confirmed nephritis
SLE 40	F	African	Yes	Father with confirmed discoid lupus/membranous nephritis
SLE 55	F	African	No	Mother with confirmed nephritis
SLE 56	F	African	No	Sister with confirmed nephritis
SLE 95	F	African	No	Sister with confirmed nephritis
SLE 112	F	African	No	Sister with confirmed nephritis
SLE 127	M	African	No	Sister with confirmed SLE
SLE 128	M	South Asian	Yes	Mother with confirmed nephritis
SLE 131	M	South Asian	Yes	Brother with confirmed nephritis,
SLE 132	M	South Asian	Yes	Brother with confirmed nephritis,
SLE 139	M	African	Yes	Sister with confirmed nephritis
SLE 143	M	East Asian	Yes	Brother with confirmed nephritis
SLE 146	M	East Asian	Yes	Brother with confirmed nephritis
SLE 153	F	African	No	Sister with confirmed nephritis
SLE 161	F	African	No	Sister with confirmed nephritis

All five patients who reported a second degree family history of SLE were female. Two were European, one was African, one was East Asian and one was of other ancestry. Two of these patients had juvenile-onset disease. Three of these patients reported non-renal SLE in aunts but the clinical details have not been confirmed. A further patient reported SLE in an uncle and another stated her grandmother had SLE, the clinical details of these relatives have not been confirmed by our study group. Four of the five patients who reported a third degree family history of SLE were female. Four patients were African and one was European. All reported cousins with SLE but the clinical details have not been confirmed.

In second and third degree relatives with reported SLE, no cases of lupus nephritis were confirmed neither was any further individual recruited to the study on the basis of self-reported information. We then carried out a clinical comparison of demographic and phenotypic data of lupus nephritis patients with a confirmed first degree family history and sporadic cases of lupus nephritis. Individuals with a self-reported second or third degree family history were excluded from this analysis.

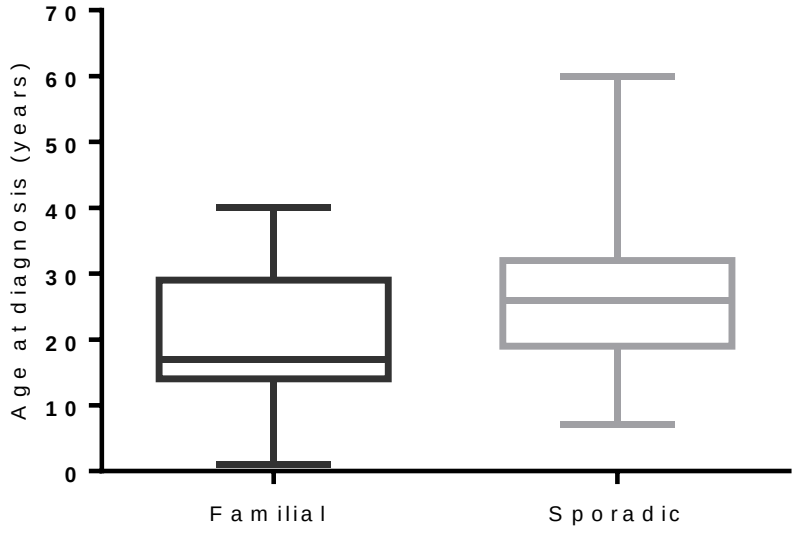
2.6.4 Clinical comparison of familial and sporadic onset nephritis

56% (n=9) of those with a confirmed first degree family history of lupus nephritis were female as compared to 88% (n=121) of sporadic cases (***p*=0.0042). Familial cases were younger (Figure 2.3) with 50% (n=8) of juvenile onset as opposed to 22% (n=30) in the sporadic onset group (**p*=0.027). 31% (n=5) and 16% (n=22) of patients were diagnosed before age 16 years in the familial and sporadic groups respectively (*p*=0.160). 13% (n=2) of the familial nephritis patients were diagnosed before 10 years of age in comparison to 1% (n=2) of the sporadic onset group (*p*=0.054).

69% (n=11) of familial cases were African while this ancestry accounted for 28% (n=38) of sporadic patients (**p=0.0015). No European patients were classified as having a first degree relative with lupus nephritis. 19% (n=3) of familial and 13% (n=18) of sporadic cases were of South Asian origin (p=0.460). 13% (n=2) of familial and 8% (n=11) of sporadic onset patients were of East Asian ethnicity (p=0.627).

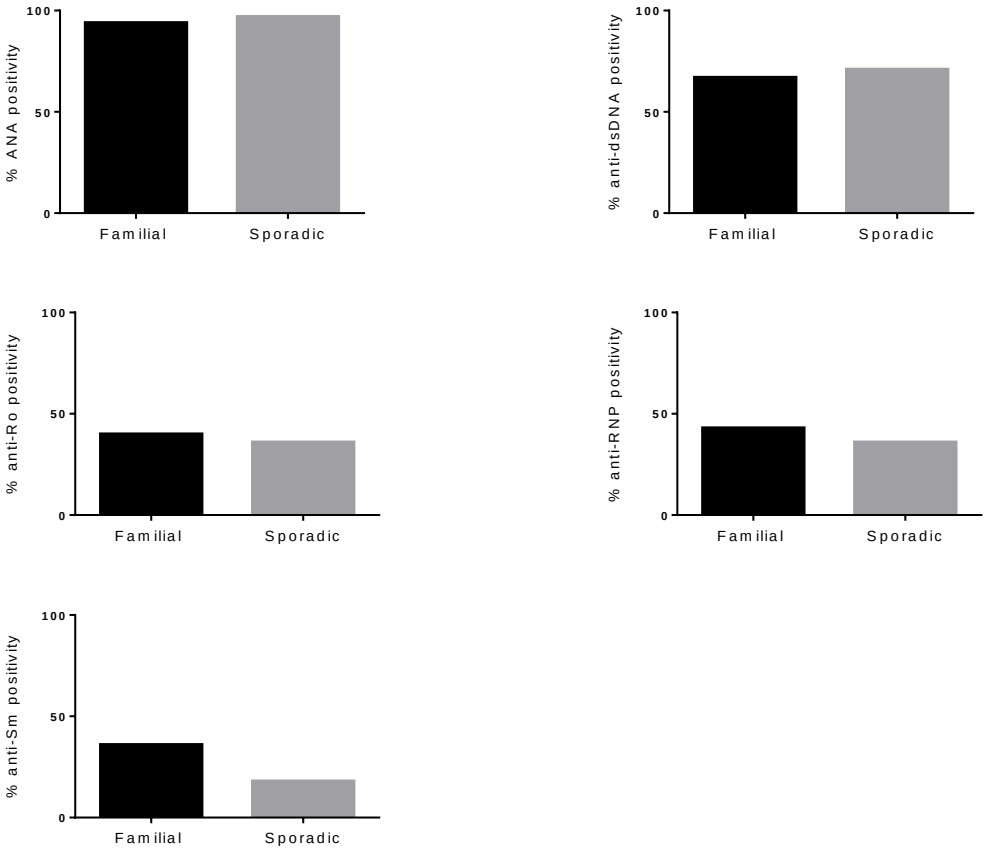
25% (n=4) of familial nephritis cases had progressed to advanced renal disease while 7% (n=9) has done so in the sporadic onset patient cohort (*p=0.032). The duration of clinical follow-up was longer in the familial group than the sporadic group, 15.1 ± 6.8 years and 11.3 ± 7.3 years respectively (p=0.054). The distribution of ISN/RPS classes in familial and sporadic lupus nephritis is available in Appendix 2.

Figure 2.3: Age of diagnosis of nephritis in familial and sporadic cases



Mean age of diagnosis of lupus nephritis in familial cases was 20.4 ± 10.6 years and 26.7 ± 11.2 years in sporadic cases (*p=0.038)

Figure 2.4: Autoantibody profile in familial and sporadic lupus nephritis



ANA positivity was 94% in familial lupus nephritis and 97% in sporadic onset disease (p=1.0). Anti-dsDNA antibodies were present in 67% of familial and 71% of sporadic cases (p=0.77). Anti-Ro were positive in 40% of familial and 36% of sporadic nephritis patients (p=0.76). Anti-RNP antibodies were seen in 43% and 36% of familial and sporadic respectively (p=0.61). Anti-Sm antibody positivity was 36% in familial nephritis and 18% in sporadic cases (p=0.15).

2.6.5 A clinical comparison of juvenile and adult onset lupus nephritis

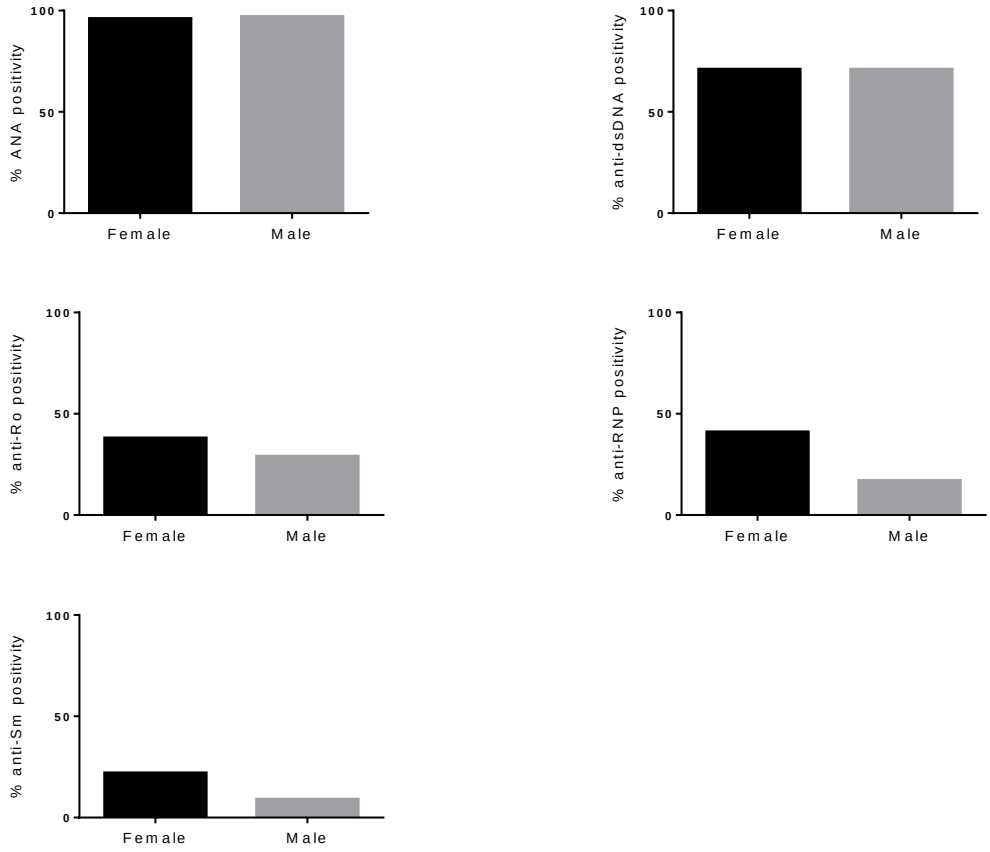
27% (n=44) of the study cohort were of juvenile onset as defined by diagnosis of nephritis before 18 years of age, while 73% (n=120) were of adult onset. 19% (n=31) were diagnosed with lupus nephritis before the age of 16 years and 2% (n=4) were diagnosed under the age of 10 years. 68% (n=30) of juvenile onset lupus nephritis patients were female as compared to 91% (n=109) of adult onset patients (***p=0.0009).

Juvenile onset patients were more likely to progress to ESRD at 16% (n=7) in comparison with 7% (n=8) of adult onset disease but this did not reach statistical significance (p=0.069). Duration of clinical follow-up was longer in the juvenile onset patients than the adult onset group, 14.2 ± 0.94 years and 10.8 ± 0.75 years respectively (*p=0.015)). 57% (n=4) of juvenile patients who developed ESRD were female and 43% (n=3) were male. 57% (n=4) were of African ancestry, 29% (n=2) were of South Asian descent and 14% (n=1) were European.

18% (n=8) of juvenile onset patients had a confirmed first degree family history of lupus nephritis while only 7% (n=8) of adult onset patients did so (*p=0.038). Juvenile patients with a first degree family history were more likely to develop advanced renal impairment than sporadic onset juveniles (*p=0.014).

30% (n=13) of juvenile onset patients and 34% (n=41) of adult patients were of African origin (p=0.577). 36% (n=16) of juvenile and 43% (n=52) of adult onset disease were European (p=0.422). South Asian ancestry was seen in 14% (n=6) of juvenile onset and 13% (n=15) of adult onset (p=0.847). 18% (n=8) of juvenile onset were of East Asian extraction as compared to 5% (n=6) of adult onset (**p=0.007).

Figure 2.5 Autoantibody profile in juvenile and adult onset lupus nephritis



The frequency of ANA was similar in both groups at 95% and 97% positivity in juvenile and adult onset respectively (p=0.51). Anti-dsDNA was present in 70% of both groups (p=0.81). Anti-Ro antibody was positive in 31% of juvenile onset cases and 38% of adult onset patients (p=0.43). Anti-RNP antibody was more frequently found in adult onset nephritis at 40% positivity as compared to 29% of juvenile onset individuals (p=0.19). Anti-Sm antibody was similar in both groups at 19% in juvenile and 20% in adult onset disease (p=0.89).

The distribution of ISN/RPS classes in juvenile and adult onset lupus nephritis is available in Appendix 3.

2.6.6 A clinical comparison of male and female nephritis

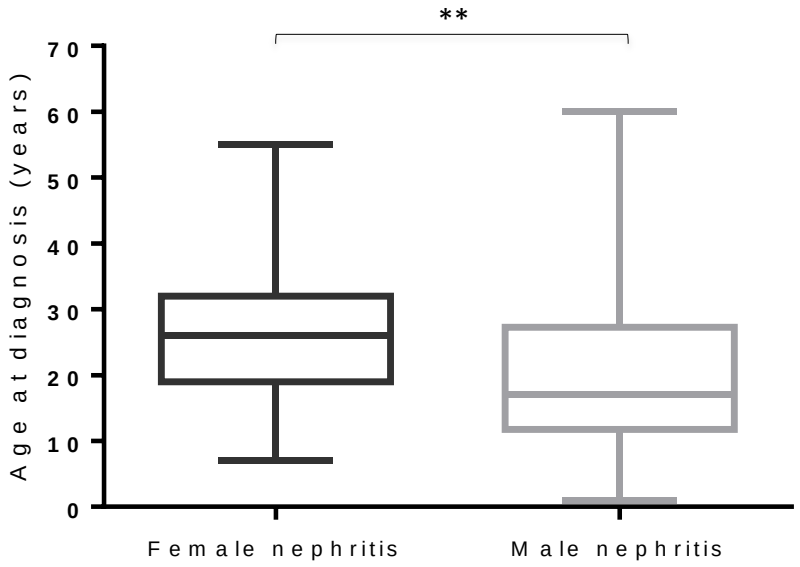
Male patients were diagnosed with lupus nephritis at a significantly younger age than their female counterparts (Figure 2.6). 56% (n=14) of males were diagnosed before the age of 18 as opposed to 21% (n=29) of females (***p=0.0008). 44% (n=11) of males were diagnosed below age 16 years as compared to 14% (n=20) of females (**p=0.0014). 12% (n=3) of males had very early onset nephritis, younger than 10 years while only 1 female presented in this age group (*p=0.011).

The mean age of female nephritis patients at study enrolment was 38.8 ± 10.9 years, while the mean current age for male patients was lower at 35.5 ± 14.8 years (*p=0.028). There was no significant difference in progression to end-stage renal failure between the genders with 9% (n=12) of females and 12% (n=3) of males reaching KDOQI stages 4 and 5 (p=0.591). The duration of follow-up between the two groups was similar, 11.5 ± 7.7 years in females and 13.2 ± 6.2 years in males (p=0.138).

28% (n=7) of male patients and 34% (n=47) of female patients were African (p=0.569). 36% (n=10) of males and 42% (n=58) of females were of European ancestry (p=0.872). 24% (n=6) of male nephritis patients and 11% (n=15) of females were of South Asian extraction (p=0.069). 8% (n=2) of males and 9% (n=12) females were of East Asian origin (p=0.917).

Confirmed first degree family history of lupus nephritis was significantly more common in male patients at 28% (n=7) when compared to females at 6% (n=9) (***p=0.0008). The distribution of ISN/RPS classes in male and female lupus nephritis is available in Appendix 4.

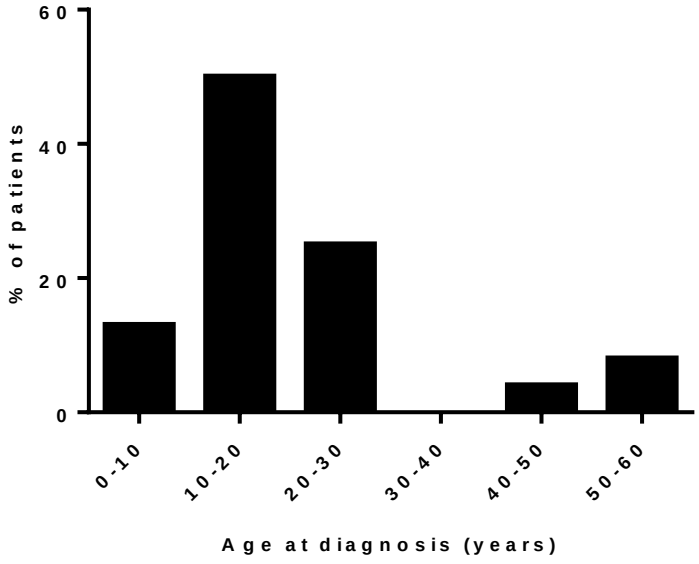
Figure 2.6 Age at diagnosis of lupus nephritis in female and male patients



Mean age at diagnosis in male lupus nephritis was 21.3 ± 14.8 years while mean age at diagnosis in females was 26.6 ± 10.2 years (**p=0.0013).

Figure 2.7 Distribution of age at diagnosis of lupus nephritis in females & males

Male



Female

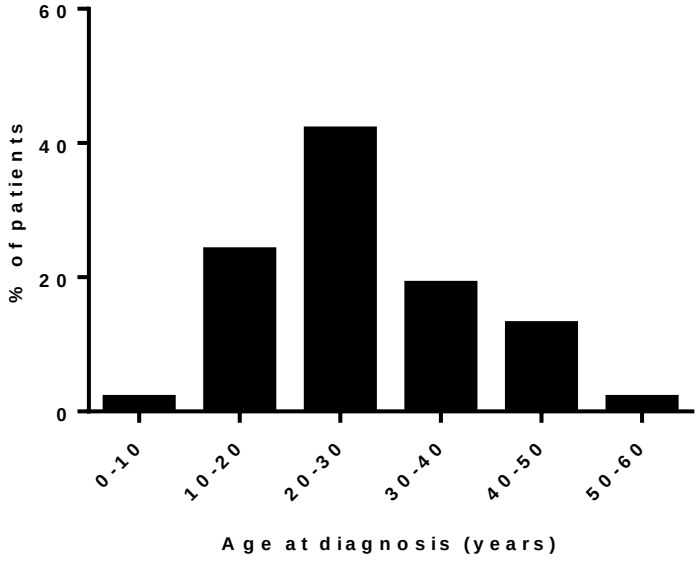
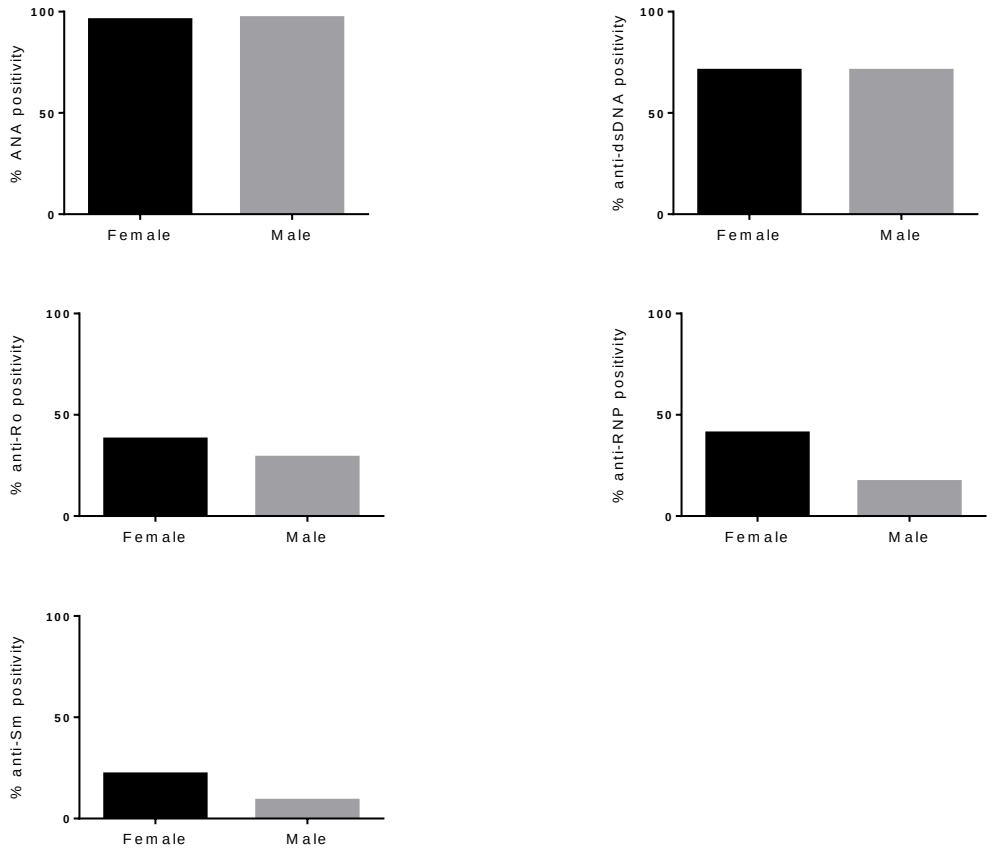


Figure 2.8 Autoantibody profile in female and male lupus nephritis



Anti-RNP antibody was more prevalent in female lupus nephritis patients at 41% as opposed to 17% in male patients (**p*=0.037). Otherwise there were no significant differences in autoantibody positivity between the genders. ANA positivity was seen in 96% of females and 97% of males (*p*=0.572). Anti-dsDNA was positive in 71% of females and males (*p*=0.981). Anti-Ro tested positive in 38% of females and 29% of males (*p*=0.436). Anti-Sm was positive in 22% of female patients and 9% of males (*p*=0.253).

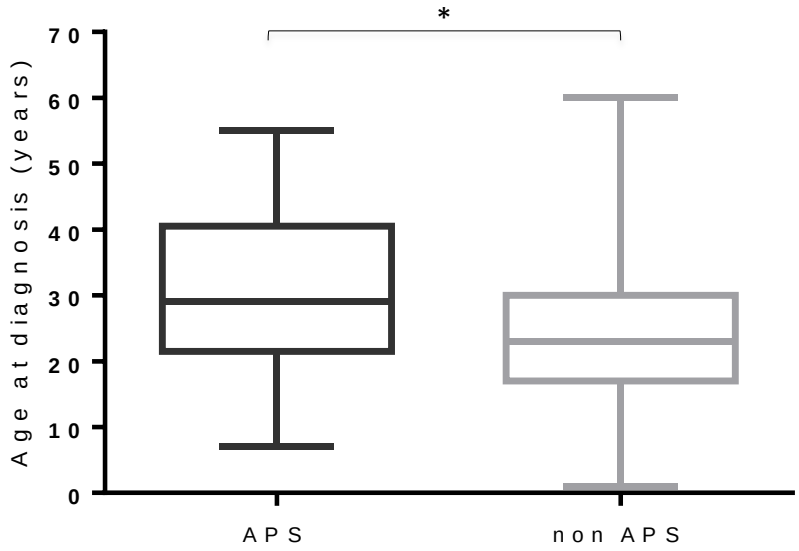
2.6.7 Bimodal distribution of disease onset in male lupus nephritis patients

A bimodal distribution of nephritis onset was noted in male patients. 84% (n=21) developed nephritis in the 2nd and 3rd decades while 16% (n=4) developed later-onset disease in the 5th and 6th decades. Mean age of diagnosis for the younger males was 16.5 ± 7.2 years and 54.2 ± 7.4 years in the older onset age group. 33% (n=7) of the younger males had a family history of SLE while none of the older males did. 14% (n=3) of the younger males have progressed to end-stage renal failure while none of the older group have done so despite similar length of follow-up between the two groups (13.5 ± 6.4 years and 11.2 ± 3.7 years respectively, p=0.436). 75% (n=3) of the older onset group were of European ancestry and 25% (n=1) was of South Asian origin. In the younger onset nephritis group 33% (n=7) were African, 33% (n=7) were European, 24% (n=5) were South Asian and 10% (n=2) were East Asian. There were no significant differences in autoantibody positivity between the two groups.

2.6.8 Antiphospholipid syndrome in lupus nephritis

16% (n=27) of lupus nephritis patients recruited to this study had a concurrent diagnosis of APS. 25% (n=41) had positive aCL and 29% (n=47) had a positive LA. 89% (n=24) of lupus nephritis patients with APS were female as compared to 84% (n=115) of those without APS (p=0.77). 4% (n=1) of those with APS and lupus nephritis had a first degree family history of lupus nephritis as opposed to 11% (n=15) of non-APS patients (p=0.48). Progression to advanced renal impairment did not significantly differ between APS and non-APS with 8% (n=2) and 9% (n=13) respectively (p=1.0). 48% (n=13) of APS patients and 42% (n=55) of non-APS were European (p=0.58). 22% (n=6) of APS and 37% of non-APS (n=48) were African (p=0.14). 19% (n=5) of APS and 12% (n=16) of non-APS were South Asian (p=0.39). 7% (n=2) of APS and (n=12) 10% of non-APS were East Asian.

Figure 2.9 Age of diagnosis of lupus nephritis in patients with and without APS



Mean age at diagnosis in lupus nephritis with APS was 30.1 ± 12.0 years while mean age at diagnosis in those without APS was 24.9 ± 10.9 years, p=0.023.

2.6.9 Progression to advanced renal impairment in lupus nephritis

To date 9% (n=15) of the study cohort have progressed to ESRD and 40% (n=6) of these have undergone renal transplantation. 80% (n=12) of those who progressed to ESRD were female and 20% (n=3) were male. 47% (n=7) were of African ancestry, 27% (n=4) were European, 13% (n=2) were of South Asian ethnicity and 13% (n=2) were of East Asian origin. 27% (n=4) had a confirmed first degree family history of lupus nephritis. 47% (n=7) were originally diagnosed with nephritis younger than 18 years of age. 33% (n=5) were diagnosed before age 16 years and 7% (n=1) before the age of 10 years. Of the nine renal biopsy samples available from these patients for our study all had proliferative nephritis, 56% (n=5) had class IV-G nephritis, 33% (n=3) had class IV-S nephritis, and 11% (n=1) had class III nephritis.

100% (n=15) were ANA positive, 75% (n=11) were anti-dsDNA positive, 27% (n=4) were anti-Ro positive, 33% (n=5) were anti-RNP positive and 27% (n=4) were anti-Sm positive. 13% (n=2) had a concurrent diagnosis of APS. 20% (n=3) had a positive aCL and 33% (n=5) has a positive LA.

2.7 Chapter Overview and Discussion

2.7.1 Lupus nephritis in different ancestral backgrounds

SLE is known to be more common and clinically more severe in non-European populations. Large multi-ancestral studies such as LUMINA (LUpus in MInorities: NAture versus Nurture) have helped characterize the role of ancestry in clinical phenotype and outcomes in SLE (Alarcon et al., 2004; Fernandez et al., 2007).

SLE patients of African ancestry have an increased frequency of lupus nephritis as compared to Caucasians (Alarcon et al., 1999; Hopkinson et al., 2000; Seligman et al., 2002). In fact European ancestry has been found to be protective against development of nephritis in SLE (Richman et al., 2012). African SLE patients experience more damage accrual and higher progression to ESRD than those of European ancestry (Dooley et al., 1997; Alarcon et al., 2001; Bastian et al., 2002; Korbet et al., 2007).

East Asian patients have been shown to have younger onset disease with a higher frequency of renal and CNS involvement (Thumboo et al., 2001; Johnson et al., 2006; Peschken et al., 2009; Golder et al., 2013). Some authors have shown increased renal and overall damage accrual in East Asian patients (Thumboo et al., 2001; Johnson et al., 2006). However a large multi-ancestral study in Canada comparing East Asian with Caucasian and African SLE patients did not find ethnicity to be predictive of damage accrual (Peschken et al., 2009).

In the United Kingdom the estimated prevalence of lupus nephritis in females is reported to be 110.3 per 100,000 in Chinese patients, 99.2 per 100,000 in Afro-Caribbean, 21.4 per 100,000 in Indo-Asian and 5.6 per 100,000 in Caucasian patients

(Patel et al., 2006). Our cohort of patients was of a multi-ancestral background with 41% of European origin, 33% of African ancestry, 13% of South Asian extraction, and 9% from East Asia. Patients of European ancestry had the latest onset of disease at 27.1 ±11.9 years. East Asian patients had the earliest onset disease at 20.9 ±9.1 years, followed by South Asians at 23.9 ±12.0 years and Africans at 26.8 ±10.7 years. In keeping with the literature Caucasian patients were the least likely to progress to ESRD at 6%, while non-European patients in our cohort had a similar frequency of advanced renal impairment of 12% approximately. It was difficult however to draw conclusions from the South and East Asian subgroups due to the limited number of patients.

In our cohort, ANA positivity was similar in European, African and South Asian patients and somewhat lower in East Asian patients at 93%. It is well established that anti-Sm and anti-RNP are more commonly seen in African SLE patients than those of European ancestry (Arnett et al., 1988; Quintero-Del-Rio et al. 2001; Cooper et al., 2002). In our patient population, anti-RNP antibodies were seen in 61% of African and 18% of Europeans. Similarly, anti-Sm antibodies were more frequent in African nephritis patients at 39% as compared to 5% of Europeans.

East Asian SLE patients have been reported to have higher anti-dsDNA, anti-Ro, anti-RNP and anti-Sm than Caucasian patients (Golder et al., 2013). Findings were similar in our cohort. Less has been published regarding autoantibody profile data in South Asian patients. ANA and anti-dsDNA prevalence has been reported to be similar in Indian SLE patients to other ethnicities (Malaviya et al., 1997). The authors also reported anti-Sm antibody positivity to be more common than in European patients but less prevalent than in Africans. Anti-Sm was least frequent in South Asian patients in our cohort at only 5%.

2.7.2 Familial versus sporadic onset lupus nephritis

An increased frequency of autoimmune disease in relatives of SLE patients is well documented in the literature. A study of 69 juvenile onset SLE patients of multiple ancestries revealed that 42% had a first, second or third degree family history of autoimmune diseases such as SLE, thyroid disease, psoriatic arthritis, rheumatoid arthritis, spondyloarthropathy, vitiligo, multiple sclerosis or type I diabetes mellitus. The most frequent autoimmune diseases seen in their relatives were SLE at 21% and thyroid disease at 15%. However having a family history of general autoimmune disease did not correlate with more severe SLE in their cohort (Walters et al., 2012). Previous work by Pluchinotta et al divided juvenile onset SLE into 3 subgroups; infantile (<age 2), pre pubertal and post pubertal. Family history of autoimmune disease was seen in 46% of infantile cases, 25% of prepubertal and 29% of post pubertal patients (Pluchinotta et al., 2007). In a study of juvenile SLE in the UK, 15% of juvenile cases had a family history of SLE and 38% had a family history of autoimmune disease in general (Watson et al., 2012).

A retrospective study, by Apenteng et al, investigated whether children with lupus nephritis and a family history of autoimmune disease had worse renal outcomes when compared with those who did not have a family history. They found that male, African patients with a family history of autoimmunity were more likely to double their serum creatinine and reach ESRD (Apenteng et al., 2006). Familial clustering of ESRD has been demonstrated in African patients with lupus nephritis (Freedman et al., 1997). Multiplex SLE families in Saudi Arabia have been shown to have earlier onset disease and higher mortality than sporadic cases (Al-Mayouf et al., 2006).

Other reports, however, do not support a more severe clinical phenotype in familial SLE cases. Multi-ancestral studies in the United States have not shown any difference in damage accrual or survival between familial and sporadic cases of SLE (Sestak et al., 2008; Burgos et al., 2010). A study of Chinese lupus nephritis patients found that clinicopathological features did not differ between familial and sporadic cases other than that class V nephritis was less common in familial disease (Wang et al., 2009). A large French study of 125 Caucasian multiplex SLE families did not show any differences in gender ratio, age of onset, clinical or immunologic features when compared to sporadic onset SLE controls (Michel et al., 2001).

69% of familial cases in our patient population were African, 19% were South Asian and 13% were East Asian. No European patients in our study had a first degree relative with lupus nephritis. In our cohort, familial cases were younger with 50% of juvenile onset as opposed to 22% in the sporadic onset group. Mean age of diagnosis in familial cases was 20.4 ± 10.6 years and 26.7 ± 11.2 years in sporadic cases. Data on frequency of family history of other autoimmune diseases was not collected in our patient group. Juvenile onset patients with a first degree family history of lupus nephritis in our study population were significantly more likely to develop advanced renal impairment than sporadic onset juvenile cases (25% versus 7%). Two of these were of African descent and two were of South Asian ancestry. Autoantibody profile was not significantly different between familial and sporadic cases nor was the distribution of proliferative and membranous nephritis between the two groups.

Overall, we found familial lupus nephritis cases to have a more severe clinical phenotype in our cohort. This may have been influenced by our strict definition of familial disease that only included first degree relatives with confirmed clinical

details and biopsy proven lupus nephritis rather than self-reported histories. Many of the studies in the literature have used a broader definition and reported on family history of autoimmune disease in general in first, second and third degree relatives. Another relevant demographic feature of our families with clustering of nephritis is that they were all of non-European ancestry, the majority being African who are known to have an increased frequency of nephritis and more severe disease.

2.7.3 Juvenile versus adult onset lupus nephritis

An estimated 20% of SLE is diagnosed in childhood or adolescence and is generally defined in the literature as disease onset before 16 years of age (Jimenez et al., 2003; Mina et al., 2010). Juvenile onset SLE typically manifests between the ages of 12 and 14 years and remains extremely rare under the age 5 years (Kamphuis et al., 2010; Watson et al., 2012). 27% of the patients in our cohort were diagnosed with nephritis before 18 years of age, 19% before the age of 16 years and 2% under the age of 10 years. The youngest patient in our cohort was diagnosed at 2 years of age.

Female preponderance in SLE is well established in adult populations with a female: male ratio of 10:1. In juvenile onset SLE this female preponderance is less pronounced with a female: male ratio of approximately 5-6:1 (Lo et al., 1999; Hiraki et al., 2008; Mina et al., 2010; Watson et al., 2012). In our cohort 68% of juvenile onset lupus nephritis patients were female as compared to 91% of adult onset patients.

Patients with juvenile onset SLE tend to present with a more aggressive disease course and a higher frequency of internal organ involvement such as nephritis and CNS disease than their adult onset counterparts. In addition, juvenile onset SLE patients are subject to more damage accrual over time leading to increased morbidity

and mortality in the long-term (Gutierrez Suarez et al., 2006; Brunner et al., 2008; Tucker et al., 2008). Lupus nephritis is more often a presenting feature of SLE in juvenile onset patients when compared to adult onset disease (Brunner et al., 2008; Tucker et al., 2008). In addition, lupus nephritis is clinically more aggressive in juvenile cases with significantly higher adjusted mean SLEDAI renal scores than in the adults with lupus nephritis (Font et al., 1998; Brunner et al., 2008; Sato et al., 2012). In our cohort juvenile onset patients were more likely to progress to ESRD than those with adult onset disease (16% versus 7%). It should be noted, however, that the duration of clinical follow-up was longer in juvenile onset than in the adult onset disease in our study (14.2 ± 0.94 years versus 10.8 ± 0.75 years).

Levy et al explored the influence of ethnicity on childhood onset SLE in a multi-ancestral cohort of patients clustered into mild, moderate and severe disease. They demonstrated that 20% of European patients were in the severe cluster as compared to 51% of Asian and 41% of African patients (Levy et al., 2013). In contrast, Miettunen et al found that neither gender nor non-European ancestry was associated with a worse clinical outcome in juvenile onset SLE patients (Miettunen et al., 2004). In our cohort, 57% of juvenile onset patients who progressed to ESRD were of African ancestry, 29% were of South Asian descent and 14% were European.

Al-Mayouf et al reported that male juvenile onset lupus nephritis had a higher progression to renal failure requiring dialysis or renal transplantation than female patients (Al-Mayouf et al., 2008). This was not the case in our patient cohort with females accounting for 57% of the juvenile onset patients who required renal replacement therapy.

A significant difference in frequency of ISN/RPS classes between juvenile and adult nephritis was not detected in our cohort. Previous studies have suggested a similar distribution of histologic classes in juvenile and adult onset lupus nephritis (Brunner et al., 2008). Marks et al showed the following distribution of ISN/RPS classes in juvenile lupus nephritis cases: 2%, 13%, 15%, 51% and 20% for classes I-V, respectively, which is similar to the findings in our group of juvenile patients (Marks et al., 2007).

The vast majority of SLE patients have positive ANA. This was true for both adult and juvenile onset patients in our cohort. Previous studies in the literature have shown that anti-dsDNA antibodies are more common and at higher titre in juvenile than adult onset SLE (Font et al., 1998; Hiraki et al., 2008; Hoffman et al., 2009). Our cohort had equal prevalence of anti-dsDNA with 70% positivity in both age groups. Anti-dsDNA antibody titres were not measured as part of our study. Anti-Ro, anti-RNP and anti-Sm antibodies are thought to be equally prevalent in juvenile and adult onset lupus (Hoffman et al., 2009). While anti-Ro and anti-RNP were more frequent in juvenile than adult onset disease in our cohort with 31% versus 38% and, 40% versus 29%, respectively, these findings not did reach statistical significance. Anti-Sm antibody was similar in both age groups in our cohort with 19% positivity in juvenile and 20% in adult onset disease.

2.7.4 The role of gender in lupus nephritis

While SLE is more common in females than males, males who develop SLE have a higher prevalence of lupus nephritis in both juvenile and adult onset disease (Kaufman et al., 1989; Molina et al., 1996; Carbone et al., 2002; Voulgari et al., 2002; Soto et al., 2004; Garcia et al., 2005). Some authors have observed an increased rate of progression to ESRD in male lupus nephritis (Specker et al., 1994;

Carbone et al., 2002) while others have not demonstrated a difference in disease severity (Miller et al., 1983; Renau et al., 2012). A multiethnic US cohort has shown that male SLE patients have a poorer long-term prognosis than females in terms of damage accrual due to accelerated damage particularly early on in their disease course (Andrade et al., 2007)

A comparison of SLE clinical data in a UK cohort over a 30-year period found a similar age of onset (29.3 years) in male and female patients (Renau et al., 2012). Male patients in their study had a different proportion of ancestral backgrounds than our cohort with 66% of European ancestry, 13% African and 18% South Asian. Our study cohort was more representative of non-European ancestries with male patients comprised of 36% Europeans, 28% Africans and 24% South Asians. In our patient population males were diagnosed with lupus nephritis at a significantly younger age (21.3 ± 14.8 years) than their female counterparts (26.6 ± 10.2 years). 44% of males in our study were diagnosed below age 16 years as compared to 14% of females. We noted a bimodal distribution of nephritis diagnosis in males. A similar distribution of disease onset in males was seen in the Renau study. 31% of males and 36% of females developed lupus nephritis in their study with 4.4% of males and 6.6% of females progressing to ESRD. In a similar fashion, we did not demonstrate a significant difference in progression to advanced renal impairment between genders in our study with 9% of females and 12% of males reaching KDOQI stages 4 and 5.

Autoantibody profile has been reported to be similar in males and females by some authors (de Carvalho et al., 2009; Renau et al., 2012) while others have documented more frequent anti-dsDNA and anti-Sm antibodies in male patients (Molina et al., 1996; Soto et al., 2004). In our cohort, anti-RNP antibody was significantly more

prevalent in female lupus nephritis at 41% as opposed to 17% in males. While anti-Ro and anti-Sm antibodies were more common in females than males (38% versus 29%) and (22% versus 9%) respectively, these did not reach statistical significance. There were similar frequencies of ANA and anti-dsDNA positivity in both genders in our study population.

2.7.5 Bimodal distribution of lupus nephritis

A bimodal distribution of nephritis onset was noted in male patients within our study group with 84% developing nephritis in the 2nd and 3rd decades and 16% later on in the 5th and 6th decades. A review of 47 patients with SLE onset after the age of 50 years from a tertiary referral centre combined with analysis of a further 714 cases from the literature found lower disease severity and a less pronounced female: male ratio (4:1) in older onset patients. The authors also demonstrated that deaths occurred more frequently in late-onset patients, with a 10-year survival rate of 71% as compared to 95% in early-onset disease, although the ultimate causes of death were usually unrelated to SLE (Boddaert et al., 2004). 14% of the younger males in our cohort have progressed to ESRD while none of the older group of males have done so despite similar length of follow-up in the two groups (13.5 ± 6.4 years and 11.2 ± 3.7 years respectively). Two females were diagnosed with lupus nephritis over the age of 50 years in our cohort and both have well maintained renal function. The majority of older-onset patients in our study were of European ancestry.

2.7.6 APS in lupus nephritis

Anti-cardiolipin antibodies and lupus anticoagulant were positive in 25% and 29% of the cohort respectively, figures that are comparable to the literature (McNeil et al., 1992). 16% of lupus nephritis patients in our study had a concurrent diagnosis of

APS. Previous studies have described the association between Antiphospholipid Syndrome Nephropathy (APSN), and hypertension, higher serum creatinine and more severe interstitial fibrosis on biopsy. Classic findings of APSN include acute thrombotic microangiopathy, arterial fibrous intimal hyperplasia and focal cortical atrophy (Nochy et al., 1999). Studies of European lupus nephritis patients have failed to demonstrate more rapid progression to end-stage renal failure in those with co-existent APSN (Daugas et al., 2002; Tektonidou et al., 2004). A study of Thai SLE patients found an association between APSN and poor renal outcome (Cheunsuchon et al., 2007). Patients in our cohort with APS in addition to lupus nephritis did not have an increased rate of developing advanced renal impairment as compared to non-APS nephritis patients. Specific histologic findings of APSN were not examined in our cohort, however.

2.7.7 Conclusion

The patient population in our study was of a multi-ancestral background. As expected, non-European lupus nephritis patients had a younger age of onset and higher rate of progression to ESRD. Familial clustering of nephritis was associated with juvenile onset disease and a more severe clinical phenotype. Several probands within these families have required renal replacement therapy. While male nephritis patients in our cohort were younger and frequently had a family history, overall they did not have an increased progression to ESRD. This outcome may have been affected by the bimodal distribution of disease onset in males and the small number of older-onset males with relatively benign disease. The next step in our genotype: phenotype analysis of lupus nephritis will be to assess if lupus susceptibility polymorphisms are more prevalent in juvenile, familial or male lupus nephritis.

Chapter 3

Histopathological findings in lupus nephritis cohort

The purpose of this chapter is to:

1. Discuss renal histology findings including ISN/RPS class of nephritis and CD68 glomerular immunostaining
2. Correlate histopathological findings with disease phenotype.

3.1 Introduction

As outlined earlier in this thesis, there is growing evidence from studies involving patients with proliferative lupus nephritis and from experiments in lupus mouse models as to the importance of monocytes in renal damage. In this chapter, we will evaluate glomerular CD68 immunostaining as a general monocytes/macrophage marker and correlate this with age at diagnosis of nephritis, gender, ancestry and other clinical parameters of clinical activity including proteinuria. We will also classify the patients enrolled in our study as per the ISN/RPS classification of lupus nephritis, introduced in 2004 to replace the previous WHO classification (Weening et al., 2004).

3.2 Study Participants

Paraffin-embedded renal biopsy tissue was available from 77% (n=126) of lupus nephritis patients recruited to this study. Biopsies were traced back to the time of the patients' original diagnosis of lupus nephritis (n=107) or when this was not possible biopsies taken at the onset of a new nephritis flare before induction immunosuppression was commenced were obtained (n=19). During analysis of immunostaining, slides with < 7 glomeruli present were excluded.

3.3 Clinical Variables

Histologic variables included NIH (National Institutes of Health) activity index (AI), chronicity index (CI) and ISN/RPS class of lupus nephritis. CD68 immunostaining in the glomerular tuft was examined as a general monocyte/macrophage marker. Urinary protein-creatinine ratio (uPCR) at the time of renal biopsy was examined when results were available.

3.4 Statistical Analysis

Descriptive statistics (mean, SD) were calculated to compare between groups. Categorical variables were compared using Pearson's Chi-squared ($\chi 2$) test, except in instances where counts were <5, when a Fisher's exact test was used. The D'Agostino-Pearson omnibus normality test was used to ascertain if data was parametrically or non-parametrically distributed. Student's t-test or Wilcoxon rank sum tests were used to compare continuous variables that were normally or non-normally distributed, respectively. Correlation between two quantitative variables, for example, CD68 count and urinary protein-creatinine ratio was assessed using Pearson's correlation coefficient, r. A value of $p<0.05$ was considered to be significant.

3.5 Materials and Methods

3.5.1 Histological techniques with renal biopsy tissue

Paraffin-embedded renal biopsy slides from transplant rejection patients were available and used to optimise immunostaining protocols prior to trial immunostaining of lupus nephritis renal biopsies. Unfortunately co-staining of lupus nephritis slides with CD68 and CD16 did not consistently provide satisfactory images for accurate quantification of cell counts in the glomerulus by confocal imaging. Given the limited amount of renal tissue that was available, it was decided to proceed to light microscopy for quantification of CD68 in the glomerulus.

3.5.2 Confocal Microscopy

Renal biopsy slides were de-paraffinised using a xylene de-waxing protocol. Slides were then rehydrated with decreasing percentages of ethanol (100%, 70% 50%). Antigen retrieval was performed using trisodium citrate (2.94g in 1L deionized

water, pH 6.0) where the slides were brought to boiling point in a microwave. Goat IgG at a 1/60 dilution was used as a blocking antibody and incubated for 30 minutes at room temperature. Primary mouse monoclonal antibodies CD68 (IgG1) at 1/100 dilution or CD31 (IgG1) at 1/50 dilution and CD16 (IgG2a) at 1/50 dilution were incubated for 1 hour at room temperature. Purified mouse IgG1 and IgG2a were used as isotype controls. DyLight 488 goat anti-mouse IgG1 and DyLight 549 goat anti-mouse IgG2a were used as secondary antibodies. Slides were mounted with Prolong-Gold Anti-fade with DAPI at a 1/300 dilution. Coverslips were placed on the slides and allowed to dry thoroughly overnight prior to confocal imaging.

A Leica SP5 microscope was used to obtain images. The 10x dry, the 20x dry and 40x oil objectives were used, with 0.4, 0.5 and 1.25 numerical apertures. Images were acquired with a resolution of 1024 x 1024 or 512 x 512 pixels and a scan speed of 200 or 400Hz. All confocal images were acquired and analysed using Leica LAS-AF software.

3.5.3 CD68 immunostaining

Slides were deparaffinised with xylene as described previously and a Tris buffer antigen retrieval protocol was followed. Mouse anti-human CD68 (Dako, KP-1) was used. Sections were then counterstained with haematoxylin. Once mounted and dried, the slides were scanned and the number of CD68 positive cells in the glomerular tuft was quantified using a virtual microscope, Pannoramic Viewer (3DHISTECH). The surface area of the tuft was used to correct for glomerular size using the Pannoramic Viewer software. This analysis was carried out by Dr Suzanne Wilhelmus, Leiden University, Holland.

3.5.4 ISN/RPS classification

ISN/RPS classification subdivides lupus nephritis into 6 classes (Weening et al., 2004):

Class I: Minimal mesangial disease

Class II: Mesangial proliferative lupus nephritis

Class III: Focal lupus nephritis (<50% of glomeruli involved)

Class IV is when >50% of glomeruli are involved and is subdivided into 2:

Class IV-G: diffuse global lupus nephritis, when ≥50% of the involved glomeruli have global lesions.

Class IV-S: diffuse segmental lupus nephritis, when ≥50% of the involved glomeruli have segmental lesions.

Class V: Membranous lupus nephritis

Class VI: Advanced sclerotic lupus nephritis

3.5.5 Activity and chronicity indices

All lupus nephritis biopsies were independently reviewed for ISN/RPS class by two of three renal histopathology collaborators, Professor Terry Cook, Dr Ingeborg Bajema and Dr Suzanne Wilhelmus. Lupus nephritis biopsies with discordant classification were additionally reviewed at a joint meeting of the involved collaborators and a consensus class was reached for each patient.

The NIH AI and CI are composite histologic scores of the active and chronic findings on renal biopsy in lupus nephritis (Austin et al., 1983). The AI reflects activity and potentially reversible changes and is scored from 0-24. The AI parameters of glomerular activity include cellular proliferation, fibrinoid necrosis

(karyorrhexis), cellular crescents, wire loop lesions (hyaline thrombi) and leucocyte infiltration. The AI parameter of tubulointerstitial activity is mononuclear cell infiltration. All AI parameters score 3 points each with the exception of fibrinoid necrosis and cellular crescents which score 6 points each. The CI reflects chronic irreversible changes and is scored from 0-12. There are 4 parameters each scoring 3 points each. The CI parameters of glomerular chronicity include glomerular sclerosis and fibrous crescents. The CI parameters of tubulointerstitial chronicity are tubular atrophy, and interstitial fibrosis.

3.6 Results

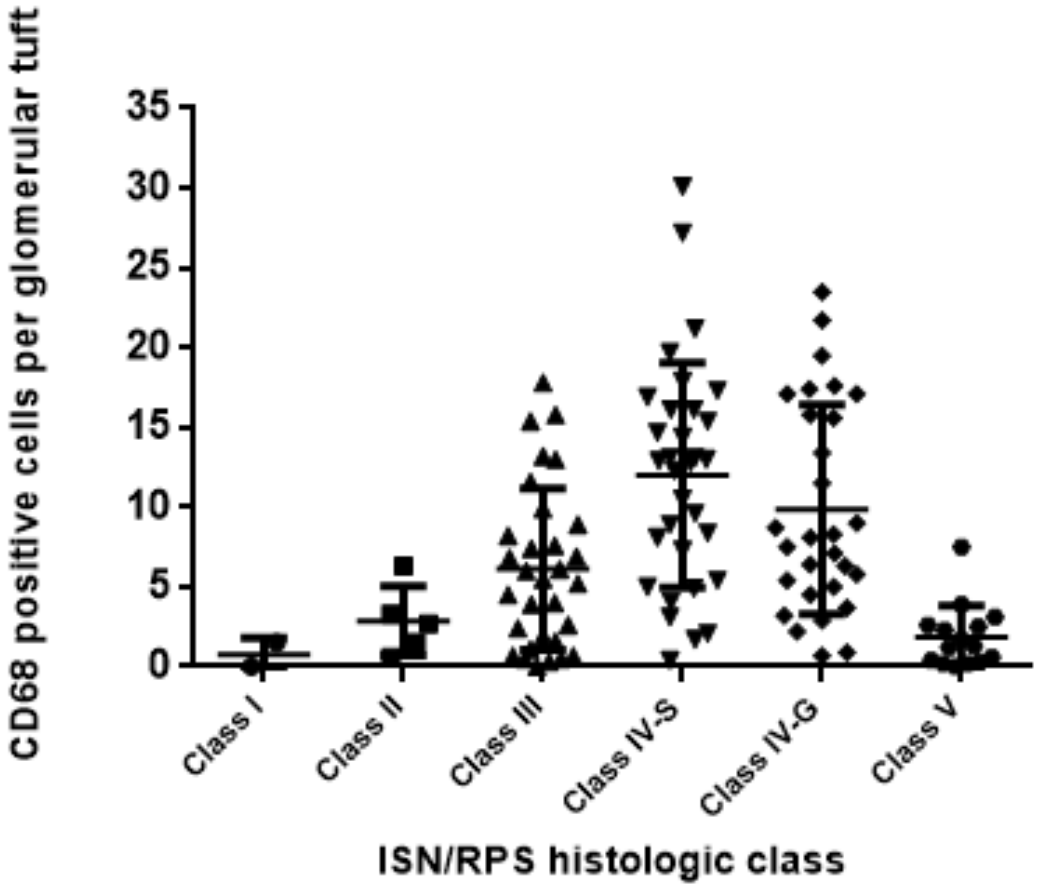
3.6.1 ISN/RPS classification in lupus nephritis

Renal biopsy slides with <7 glomeruli were excluded from the analysis, leaving 117 biopsies for CD68 analysis and 114 biopsies for ISN/RPS classification. 85% (n=97) of biopsies were concordantly classified by both renal histopathologists. Discordant ISN/RPS classification occurred in 15% (n=17) of cases. Of these 42% (n=8) were due to class IV-G/class IV-S discordance, 32% (n=6) were class IV-S/class III discordance and 16% (n=3) were due to class IV-G/class III discordance. Following review of these 17 cases at a consensus meeting, a final decision was made on ISN/RPS class for these individuals.

2% (n=2) of the study group were classified as ISN/RPS class I, minimal mesangial disease. 4% (n=5) were classified as class II, mesangial proliferative lupus nephritis. 27% (n=31) were class III, focal proliferative lupus nephritis. 25% (n=28) were classified as class IV-G, diffuse global lupus nephritis. 28% (n=32) were class IV-S, diffuse segmental lupus nephritis. 14% (n=16) had 'pure' class V. In addition, 7% had proliferative nephritis overlapping with class V, of these 2 had class IV-G, 3 had class IV-S and 3 has class III. There were no biopsies showing class VI, advanced sclerotic lupus nephritis.

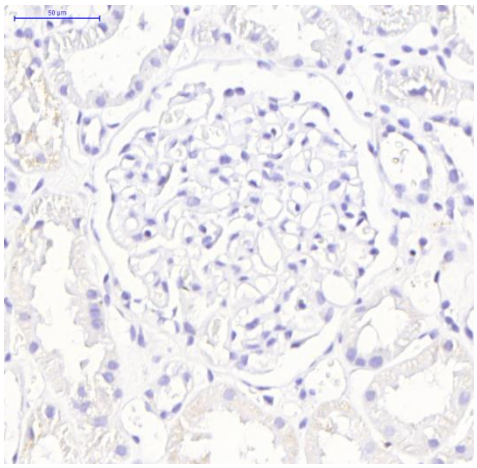
3.6.2 CD68 immunostaining results

Figure 3.1: CD68 positivity in ISN/RPS histologic classes

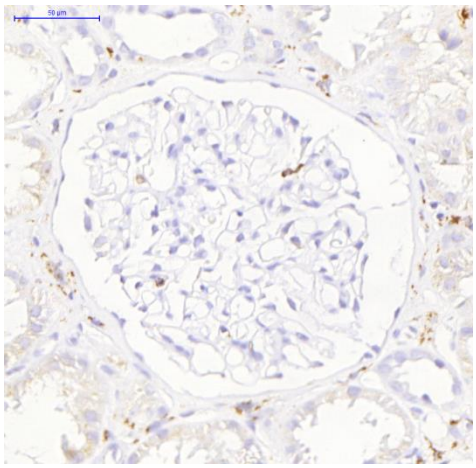


Class IV-G had significantly higher glomerular CD68 than classes I, II, III and V (*p=0.05, *p=0.027, *p=0.017, ****p<0.0001 respectively. Class IV-S had significantly higher CD68 enrichment than classes I, II, III and V (*p=0.034, **p=0.007, ***p=0.0004, ****p<0.0001 respectively. There was no significant difference in the number of CD68 positive cells between classes IV-S and IV-G. Given the low CD68 staining in classes I, II and V, these classes were then excluded from further histopathological correlation with clinical parameters.

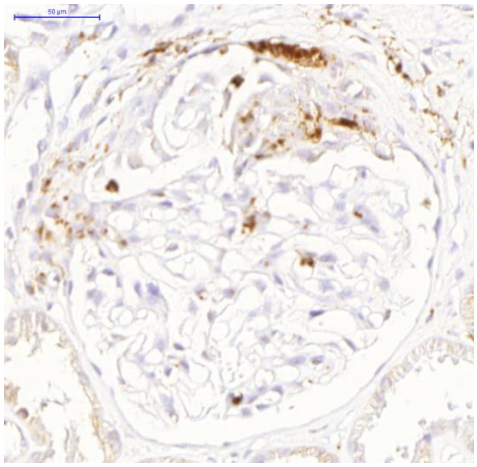
Figure 3.2: Representative images of CD68 staining in ISN/RPS classes



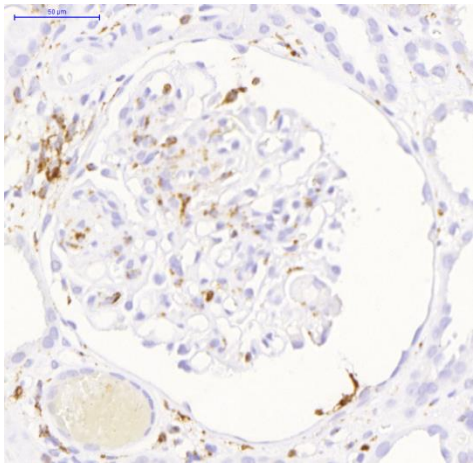
Class I



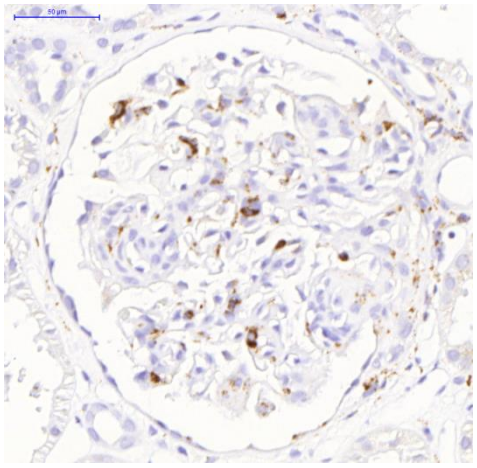
Class II



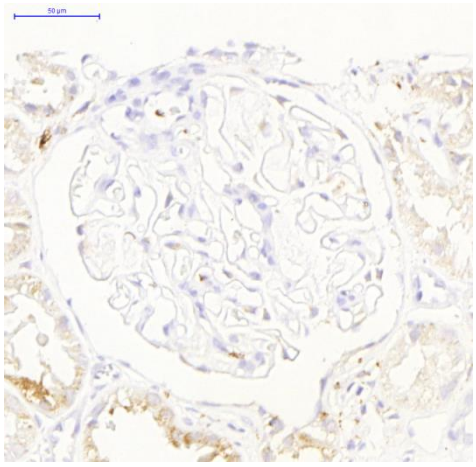
Class III



Class IV-S

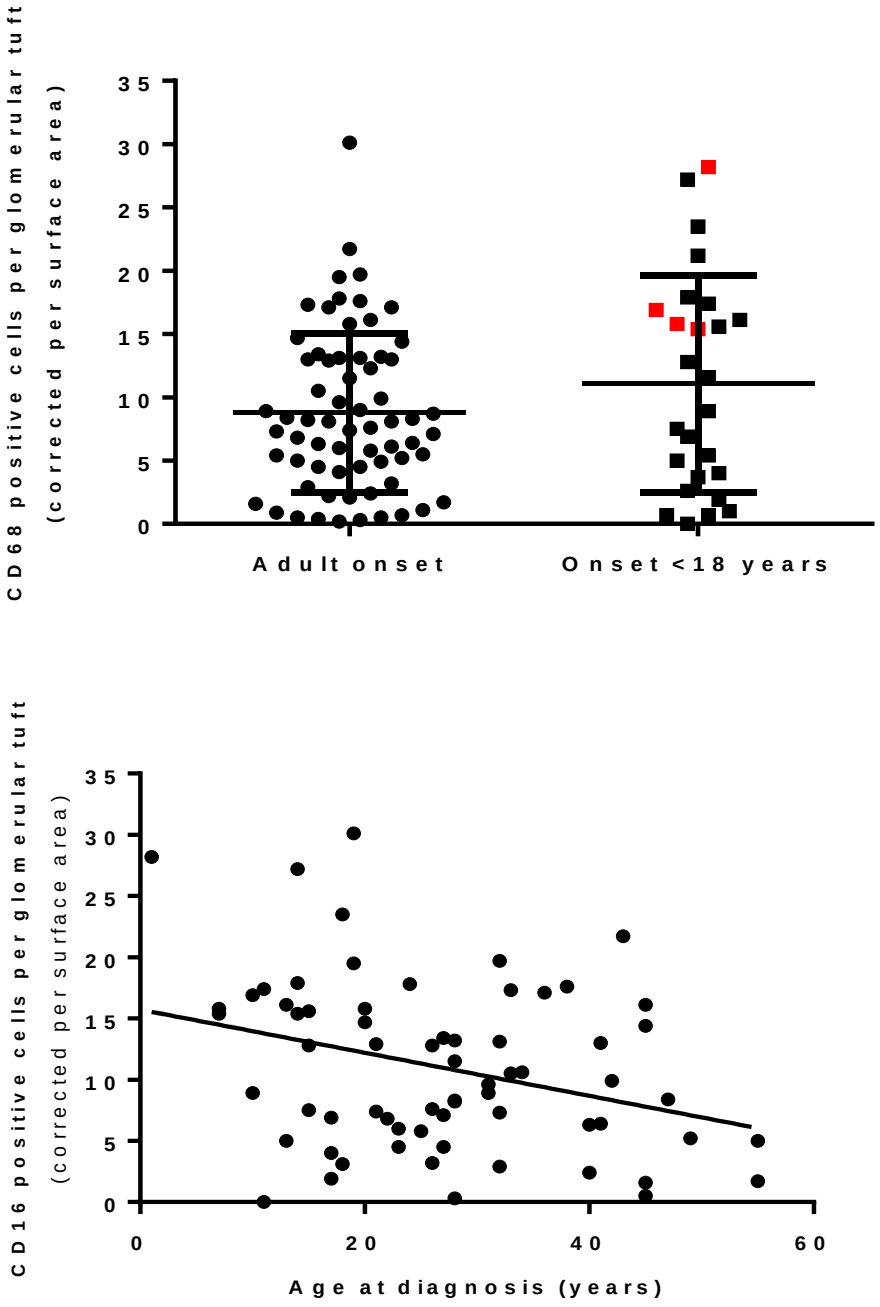


Class IV-G



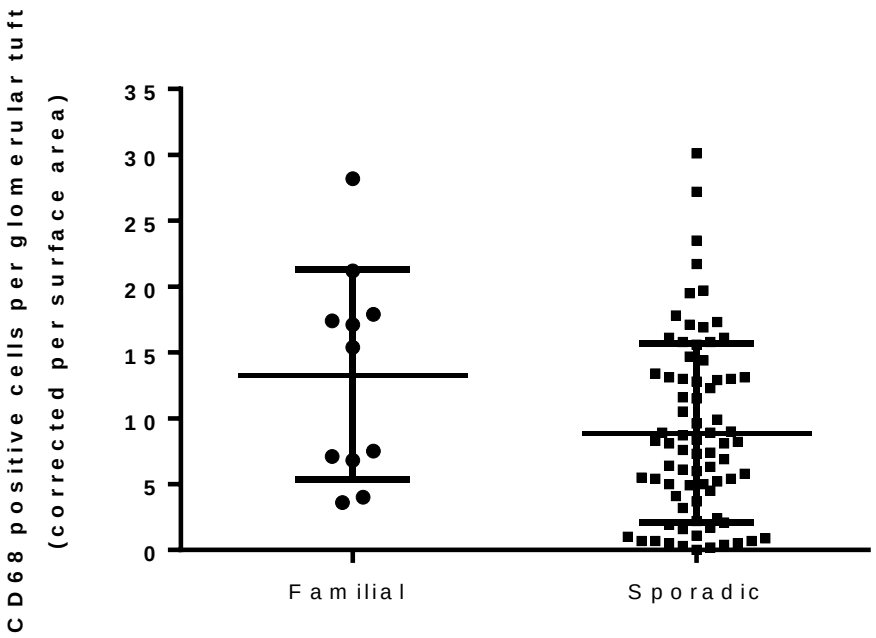
Class V

Figure 3.3: CD68 positivity in proliferative lupus nephritis comparing juvenile and adult onset nephritis



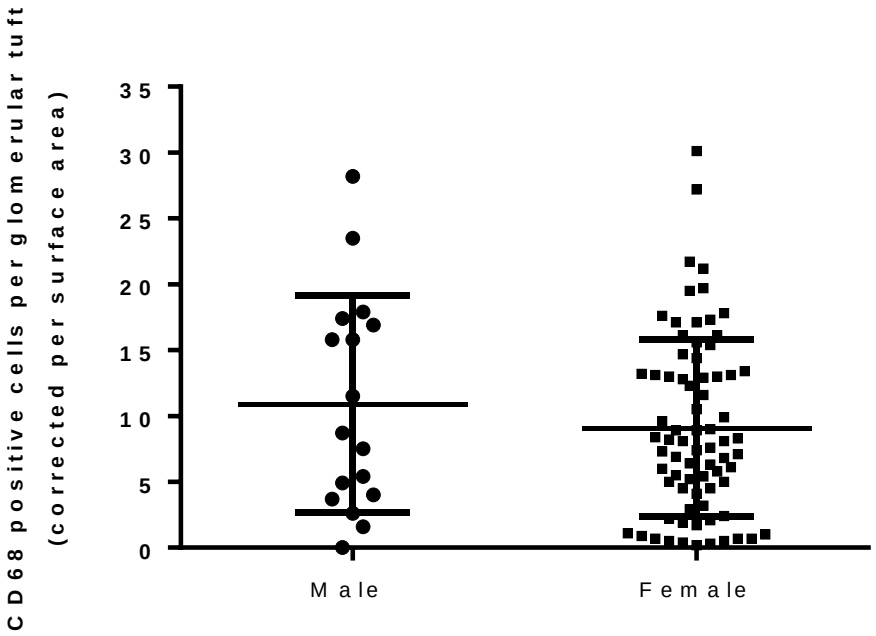
The mean CD68 count in adult onset nephritis was 8.8 ± 6.3 and 11.1 ± 8.5 in juvenile onset disease (p=0.37). Classes I, II and V were excluded from the analysis. Red indicates lupus nephritis patients with diagnosis before age 10 years. Glomerular CD68 count correlated with age at diagnosis of nephritis (r=-0.314, **p=0.0096).

Figure 3.4: CD68 cell positivity in proliferative lupus nephritis comparing familial and sporadic disease



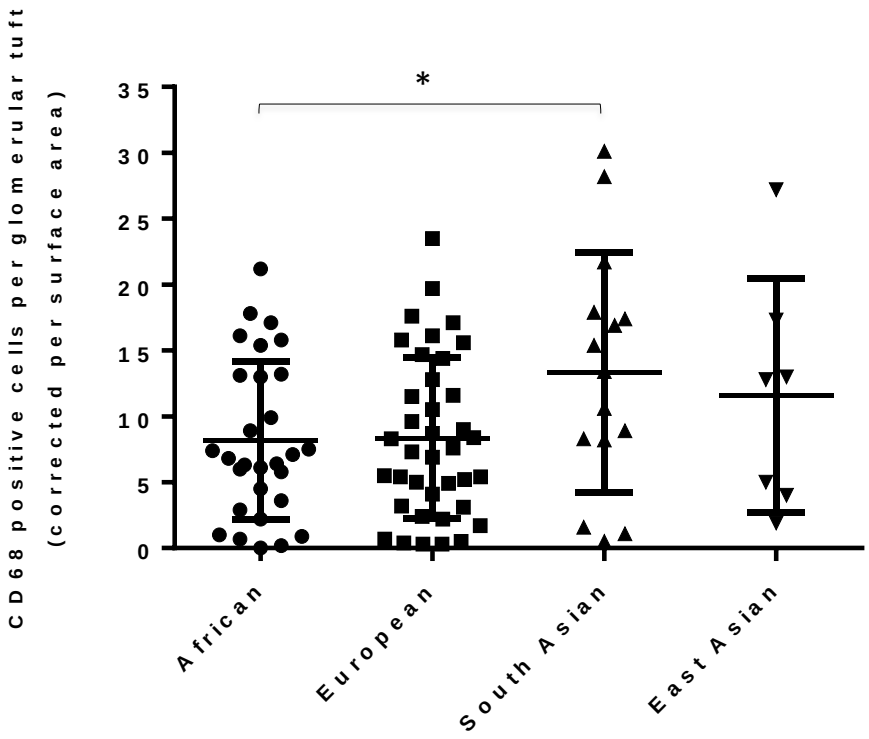
The mean glomerular CD68 count in familial nephritis was 13.3 ± 8.0 and in sporadic cases was 8.9 ± 6.8 (p=0.07). Classes I, II and V were excluded from the analysis.

Figure 3.5: CD68 cell positivity in proliferative lupus nephritis comparing male and female disease.



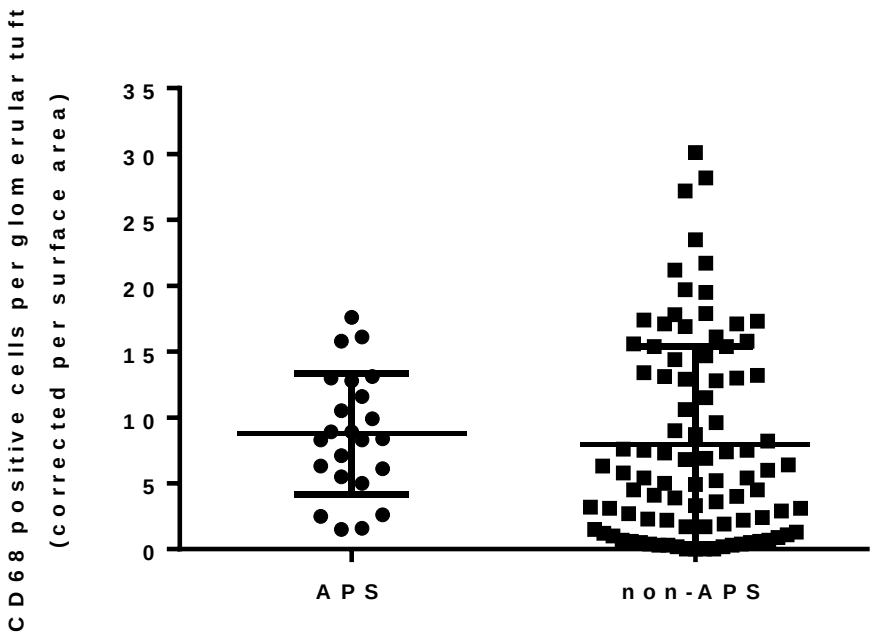
The mean glomerular CD68 count in males was 10.9 ± 8.3 and 9.1 ± 6.7 in females (p=0.49). Classes I, II and V were excluded from the analysis. When analysed excluding males with onset 5th to 6th decades results were also non-significant.

Figure 3.6: CD68 cell positivity in proliferative lupus nephritis as per ancestry



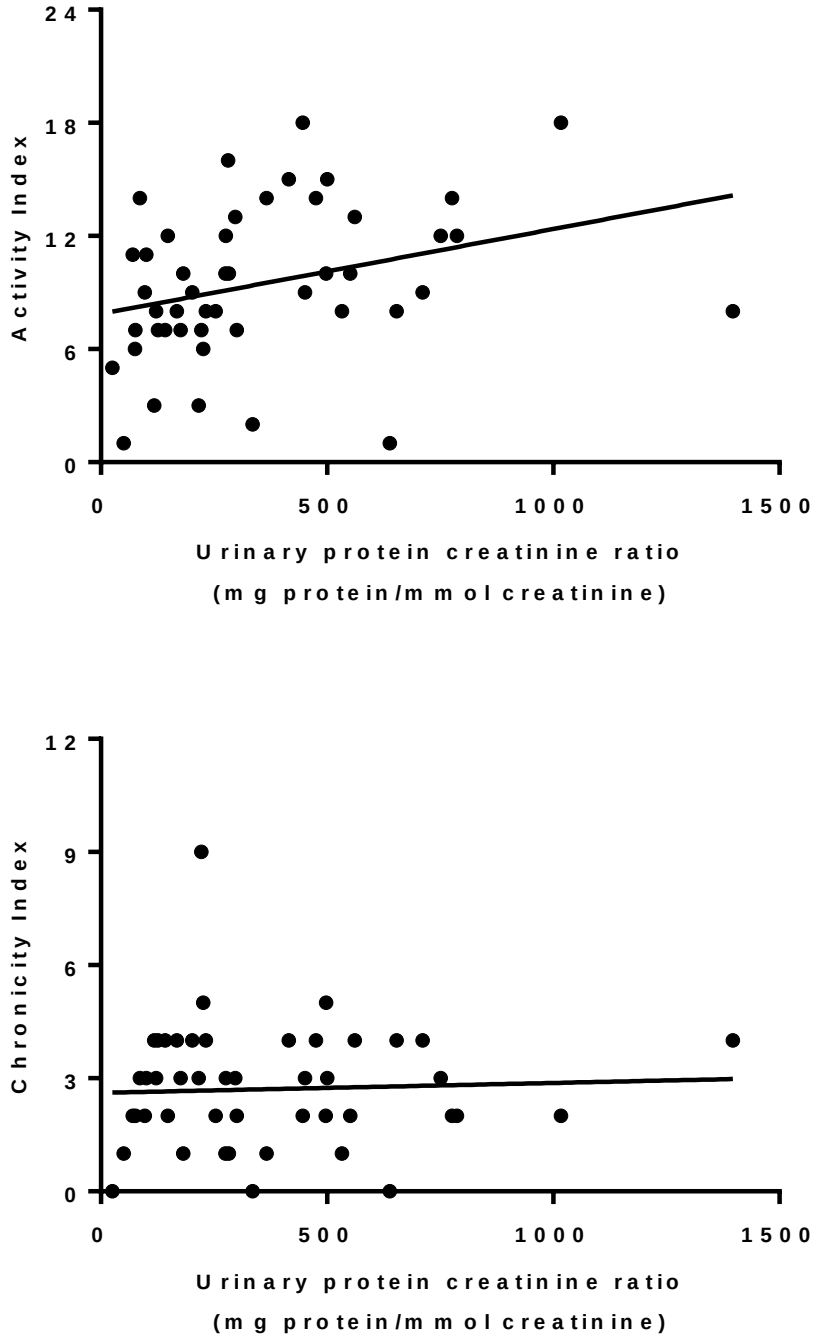
The mean glomerular CD68 count in European, African, South Asian and East Asian patients were 8.2 ± 5.9, 8.3 ± 6.1, 13.4 ± 9.1 and 11.6 ± 8.8 respectively. Classes I, II and V were excluded from the analysis. South Asian CD68 enrichment was significantly higher than African (**p*=0.038) otherwise there were no statistically significant differences in CD68 positivity between ancestries.

Figure 3.7: CD68 cell positivity in proliferative lupus nephritis in APS



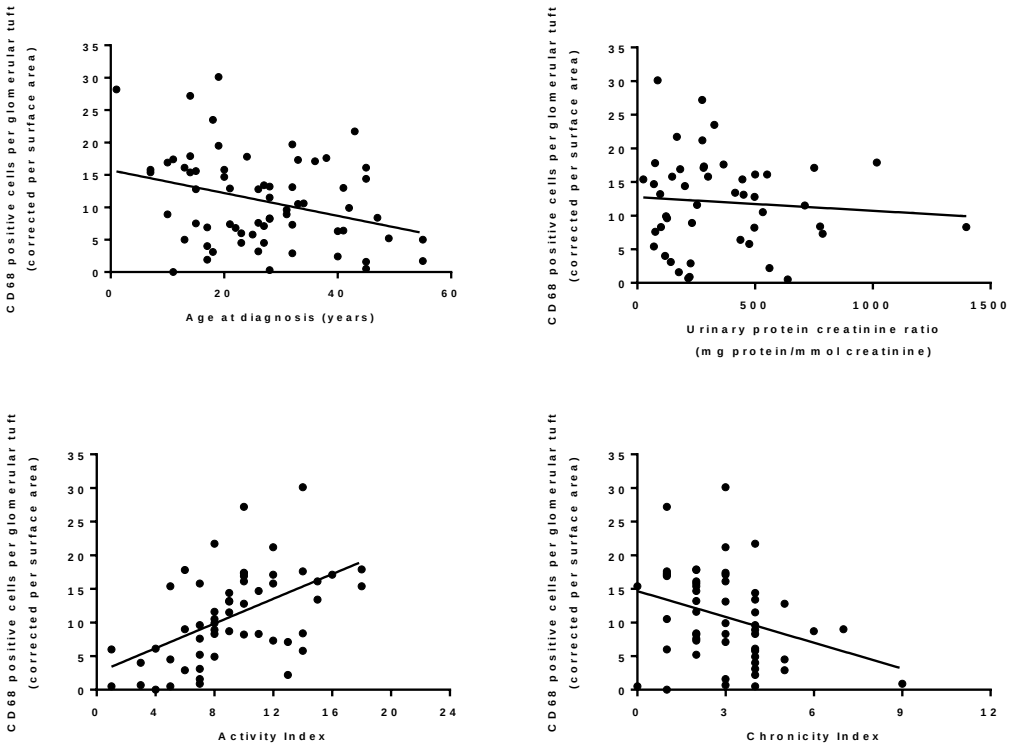
The average number of CD68 positive cells per glomerular tuft (corrected per surface area) in nephritis patients with and without APS. Mean glomerular CD68 count in APS was 8.8 ± 4.6 and 7.9 ± 7.5 in non-APS patients (p=0.15). Classes I, II and V were excluded from the analysis.

Figure 3.8: Correlation of urinary protein-creatinine ratio with activity & chronicity indices



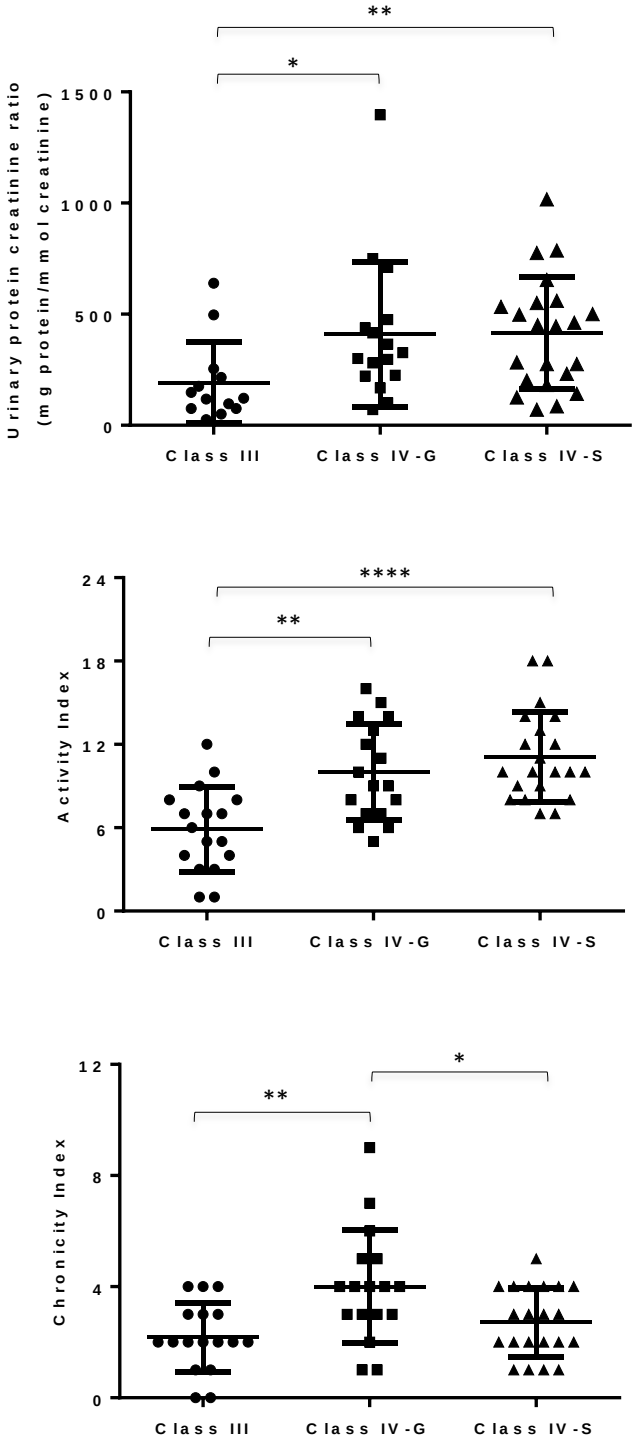
NIH AI correlated positively with uPCR: r= 0.311 (**p*=0.031). NIH CI did not correlate with uPCR: r= 0.046 (*p*=0.757).

Figure 3.9: Correlation of CD68 with age at diagnosis/proteinuria/activity index/chronicity index



Glomerular CD68 count correlated with age at diagnosis of nephritis (r=-0.314, ***p*=0.0096). There was no correlation between CD68 and uPCR (r= - 0.079, *p*=0.583). There was a positive correlation between CD68 count and AI (r= 0.525, *****p*<0.0001). A negative correlation was seen between CD68 count and CI (r= -0.3128, **p*=0.021).

Figure 3.10: Proteinuria, activity & chronicity indices in proliferative lupus nephritis



Mean uPCR was 191.8 ± 181.2 in class III, 409.0 ± 324.1 in class IV-G and 414.1 ± 252.2 in class IV-S nephritis. Class III patients had significantly lower uPCR than class IV-G and class IV-S, (*p=0.01) (**p=0.004) respectively. There was no difference in uPCR between class IV-G and class IV-S (p=0.66).

Mean AI was 5.8 ± class III, 10.0 ± 3.5 in class IV-G and 11.1 ± 3.2 in class IV-S. Class III had significantly lower AI than classes IV-G and IV-S (**p=0.0016) (****p< 0.0001) respectively. AI was similar in classes IV-G and IV-S nephritis (p=0.32). Mean CI was 2.2 ± 1.2 in class III, 4.0 ± 2.0 in class IV-G and 2.7 ± 1.2 in class IV-S. CI in class IV-G was significantly higher than in classes III and IV-S (**p=0.0034) (*p=0.02) respectively. There was no difference in CI between classes III and IV-S (p=0.19).

3.7 Chapter Overview and Discussion

3.7.1 Discussion of ISN/RPS classification

The role of the mononuclear phagocytic system in lupus nephritis has been reviewed in the introduction of this thesis. In our study cohort, we used CD68 as a general monocyte/macrophage marker and were interested in correlating CD68 immunostaining with clinical parameters in lupus nephritis. We have focused on CD68 staining in the glomerular tuft, however, while not examined in depth here, the importance of tissue resident macrophages and dendritic cells in the pathogenesis of lupus nephritis should not be minimized. In addition, tubulointerstitial disease is a well-recognized feature of lupus nephritis however CD68 immunostaining in this area was not quantified in our study.

We found that classes I, II and V nephritis had minimal glomerular CD68 infiltration and, therefore, excluded these classes from our clinical analysis. We chose, instead, to focus primarily on the proliferative classes III, IV-G and IV-S. Class III nephritis had significantly less CD68 enrichment than classes IV-G and IV-S. We did not find a significant difference in glomerular CD68 cell count between class IV-G and IV-S. This is contrary to previous work by Hill et al who showed that class IV-G nephritis had more glomerular monocytes/macrophages than class IV-S nephritis (Hill et al., 2005). Their study included 15 class IV-S and 31 class IV-G patients as compared to the 28 class IV-G and 32 class IV-S patients in our cohort. There were some potentially relevant demographic differences between the two study populations given the known higher severity of nephritis in juvenile and non-Caucasian patients. The Hill study population was comprised of 63% Caucasian, 17% North African, 8% Asian and 11% African patients. Our cohort, in contrast, had fewer Caucasian patients and more African and Asian individuals. South and East Asian patients in our study were among those with the highest CD68 scores. The age of patients who had a renal biopsy in their study population was older, with an average age of 35 years, as compared to the average age of 25 years of patients who were biopsied in our study. While age at diagnosis did not correlate strongly with CD68 count in our cohort, patients with juvenile onset disease <10 years had higher than average CD68 scores.

A number of studies have reported that AI is higher in global than segmental class IV nephritis ((Najafi et al., 2001; Yokoyama et al., 2004; Hill et al., 2005). We found similar AI scores in classes IV-S and IV-G. CI has also been reported to be higher in global than segmental nephritis (Mittal et al., 2004; Yokoyama et al., 2004; Hill et al., 2005). Our study findings were consistent with that of these authors.

Hill et al describe higher levels of proteinuria in class IV-G nephritis than class IV-S. Our study found similar urinary protein-creatinine ratios in both classes. Anti-dsDNA titres at time of biopsy were higher in class IV-G cases than class IV-S cases in the Hill and Najafi studies. We found anti-dsDNA to be more prevalent in class IV-S than IV-G nephritis cases (84% versus 75%), however data on antibody titres were not consistently available due to changes in laboratory methodology over the study period in our cohort making comparison of antibody titres difficult. We did not find any differences in CD68 cell count in patients who were autoantibody positive or negative for ANA, anti-dsDNA, anti-Ro, anti-RNP and anti-Sm. The distribution of autoantibody prevalence and ISN/RPS class is available in Appendix 5.

Although a number of retrospective studies have shown significant clinical and morphological differences between class IV-G and class IV-S nephritis, there is a lack of consensus as to which class has a worse clinical outcome. A recently published meta-analysis did not show a significant difference in renal outcome between class IV-G and IV-S using doubling of serum creatinine and development of end-stage renal failure as clinical endpoints (Haring et al., 2012). Of the nine renal biopsy samples available from patients who progressed to ESRD in our study, 56% had class IV-G, 33% had class IV-S, and 11% had class III nephritis. It is difficult to draw definitive conclusions on progression to ESRD from our data given the multitude of confounding factors that may have affected renal outcomes including the retrospective nature of the study, differences in duration of disease follow-up, differences in immunosuppressive agents used, variable degrees of hypertension, and the issue of patient compliance with medication.

3.7.2 Discussion of CD68 immunostaining results

Glomerular CD68 count correlated with age at diagnosis of nephritis (r=-0.314, ***p*=0.0096) and a subgroup of patient with disease onset before age 10 years had particularly high CD68 counts. Unsurprisingly, we found a strong positive correlation between CD68 and AI (r= 0.525, *****p*<0.0001) and a negative correlation between CI and CD68 (r= -0.3128, **p*=0.021) in our patients. The mean glomerular CD68 count was higher in familial nephritis (13.3 ± 8.0) than in sporadic cases (8.9 ± 6.8) but this did not reach statistical significance (*p*=0.07). No difference was seen in CD68 between genders, even when older onset males with relatively benign disease were removed from the analysis. The mean glomerular CD68 count was highest in South Asian patients, followed by East Asian individuals. European and African patients had similar CD68 counts.

A number of variables need to be mentioned that may have influenced CD68 count, including the time duration between subclinical nephritis onset and biopsy, the duration between diagnosis of SLE and diagnosis of nephritis at biopsy and immunosuppression at time of biopsy. Further immunostaining of our patients' renal biopsies is ongoing with CD16 in an attempt to further decipher the functional heterogeneity of the mononuclear phagocytic system and its role in lupus nephritis. Other markers are also being considered for immunostaining including CX3CR1. Analysis of CD68 immunostaining in the context of lupus susceptibility polymorphisms is discussed in Chapter 4.

Chapter 4

Genotype-phenotype relations of lupus susceptibility genes in lupus nephritis patients

The purpose of this chapter is to:

1. Establish if known lupus susceptibility polymorphisms are associated with lupus nephritis in patients of different ancestral backgrounds within our cohort.
2. Determine if susceptibility polymorphisms are associated with clinical parameters including family history of nephritis, age of disease onset, autoantibody profile and CD68 count.

4.1 An introduction to genotyping by ImmunoChip

ImmunoChip is an Illumina Custom Genotyping array containing approximately 2000,000 polymorphisms, designed to perform replication studies of GWAS in autoimmune and inflammatory diseases and fine-mapping of known GWAS hits. Development of the chip was initiated by the Wellcome Trust Case-Control Consortium and designed following contributions from research groups with an interest in diseases such as SLE, rheumatoid arthritis, ankylosing spondylitis, psoriasis, Crohn's disease, ulcerative colitis, coeliac disease, autoimmune thyroid disease, type I diabetes, primary biliary cirrhosis, primary sclerosing cholangitis, and multiple sclerosis. For each of these disorders, approximately 3,000 SNPs were chosen from available GWAS data. In addition, ancestry informative markers (AIMs) are included on ImmunoChip to facilitate identification of outliers from ancestral groups and to avoid bias caused by population stratification.

The use of ImmunoChip has enabled us to genotype many of the known lupus susceptibility polymorphisms identified by GWAS (Table 4.1) and to study them in detail, both on an individual basis and as an aggregate in a Genetic Risk Score (GRS). Firstly, we can test if individual SNPs are associated with lupus nephritis within our cohort using healthy control genotype data from the 1,000 Genomes Project. Secondly, and perhaps more relevant to clinical practice we can also test how these polymorphisms correlate with clinical findings within our cohort such as age of onset, family history, autoantibody profile and histology findings.

Table 4.1 Lupus susceptibility genes of interest genotyped by ImmunoChip

Gene	Chromosome	Genotyped SNP	Risk Allele	OR
IRF5	7q32	rs4728142 rs2070197	A C	1.43 1.88
IRAK1	Xq28	rs2269368	T	1.11
PTPN22	1p13.2	rs2476601	A	1.35
ITGAM	16p11.2	rs1143679 rs9888739 rs4548893	T T A	1.40 1.62 1.34
IRF7	11p15.5	rs4963128	A	1.33
IRF8	16q24.1	rs2280381	A	1.17
STAT4	2q32	rs7574865 rs3821236	T A	1.77 1.49
NCF2	1q25	rs10911363	T	1.18
IKZF1	7p12.2	rs4917014	C	1.39
IFIH1	2q24	rs1990760	T	1.23
TNFAIP3	6q23.3	rs6920220 rs2230926	A C	1.17 1.72
ETS1	11q24.3	rs6590330	A	1.37
HLA-DRB1	6p21.32	rs3135394 rs9271366	G G	1.98 1.26
BLK	8p23.1	rs2736340	T	1.38
BANK1	4q24	rs10516487	G	1.38
TNFSF4	1q25.1	rs2205960	A	1.46
RasGRP3	2p22.3	rs13385731	G	1.43
LYN	8q12.1	rs7829816	C	1.30

4.2 Methods

4.2.1 Genotyping by ImmunoChip

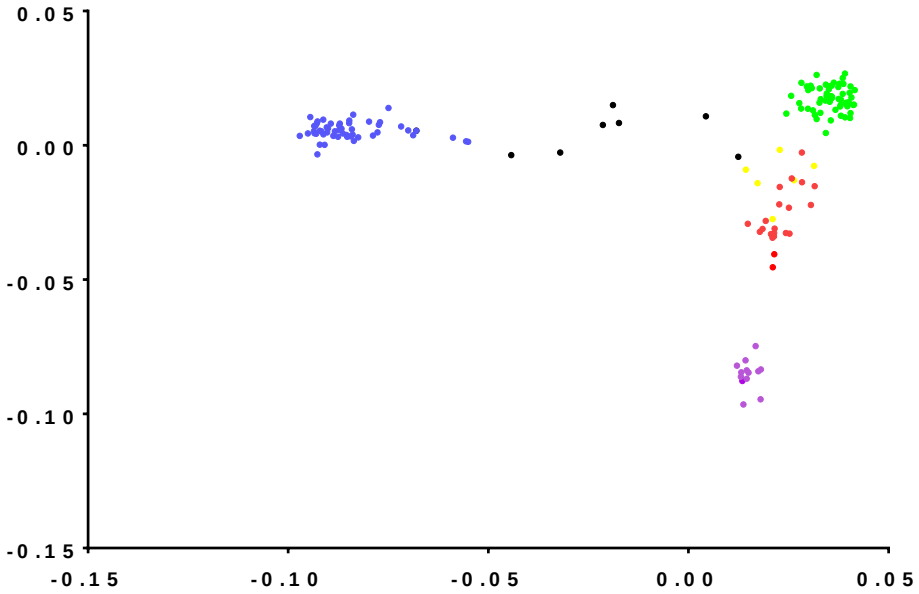
Genomic DNA was extracted from thawed frozen whole blood using the GenElute™ Blood Genomic DNA Kit (Sigma Aldrich) as per the manufacturer's instructions. Genotyping was performed using the Illumina ImmunoChip with the assistance of Dr Urmas Roostalu in the Genomics Facility, 7th Floor, Guy's Tower, Guy's Hospital. Analysis and genotype calling was performed using Illumina GenomeStudio software. Of 196,524 polymorphisms available on ImmunoChip, 184,962 passed quality control and were suitable for analysis.

4.2.2 Study Participants

164 patients with biopsy-proven lupus nephritis were genotyped by ImmunoChip. 4 patients' genotyping results did not meet quality control standards leaving 160 patients results suitable for analysis. Population stratification is a major consideration when analysing association studies as genetic allelic frequencies differ significantly between different ancestral groups and may be a confounding factor leading to false positive results. To deal with this issue of population stratification, Multidimensional Scaling (MDS) was carried out using PLINK (Purcell et al., 2007).

Complete linkage clustering on the basis of autosomal genome-wide SNP data was performed based on IBS (identity-by-state) distance between individuals. This subdivided the study group into clusters, in our study these clusters were named; clusters 1, 2 and 3. Multidimensional Scaling (MDS) analysis was then performed on the matrix of genome-wide IBS distances. Plotting each cluster's values against another resulted in a scatter plot on which each point was an individual. When all 3 clusters were ultimately plotted against one another, it enabled us to identify outliers from the main ancestral groups.

Figure 4.1: Identifying population outliers by Multidimensional Scaling



Cluster 1: African (n=54)

Cluster 1 (denoted in blue) were patients of African ancestry (Afro-Caribbean, Nigeria, Ghana, Sierra Leone, and Uganda).

Cluster 2: European (n=62)

Cluster 2 (denoted in green) were patients of Northern European ancestry (United Kingdom and Ireland).

Cluster 3: East Asian (n=14)

Cluster 3 (denoted in purple) were patients of East Asian ancestry (China, Vietnam, and Singapore).

Population Outliers from the main 3 clusters

Black denotes patients who were classified as outliers from the main ancestral groups. Red denotes patients who reported being of South Asian ancestry (India and Pakistan) (n=21). Yellow denotes patients from the following countries: Spain, Portugal, Chile, Columbia and Brazil (n=6). Two of these patients were from countries in the Middle East and are denoted by the 2 points nearest to the European cluster. Five patients reported parental admixture. Of these; 3 had an African parent and a European parent, 1 had an African parent and an East Asian parent and 1 had a European parent and an East Asian parent.

On the basis of this MDS, genotyping results from the 3 distinct ancestral clusters (African, European and East Asian) were compared with 1,000 genomes data from the same ancestry for frequency of risk alleles of lupus susceptibility polymorphisms. Patients with Hispanic ancestry were excluded from the European group due to their position outside the main genetic cluster. When exploring the association of clinical parameters with a particular polymorphisms risk allele, all patients were included regardless of ancestry or being a population outlier.

4.2.3 Statistical Analysis

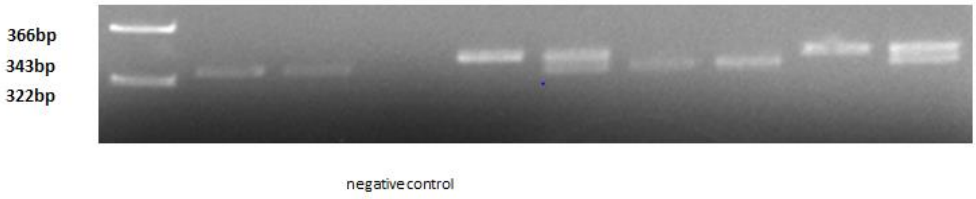
The difference in allelic distribution between lupus nephritis patients and 1,000 genomes control data was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. Continuous variables were described as mean with standard deviation (SD). Categorical variables were compared using Pearson's Chi-squared ($\chi 2$) test, except in instances where counts were <5, when a Fisher's exact test was used. The D'Agostino-Pearson omnibus normality test was used to ascertain if data was parametrically or non-parametrically distributed. Student's t-test or Wilcoxon rank sum tests were used to compare continuous variables that were normally or non-normally distributed, respectively. Correlation between two quantitative variables was assessed using Pearson's correlation coefficient, r. A value of $p<0.05$ was considered to be significant. Analysis was carried out using GraphPad Prism 6 software.

4.2.4 FCGR2A H/R131 polymorphism genotyping by allele-specific restriction enzyme digestion (ASRED)

Genomic DNA was extracted from thawed whole blood using the GenElute™ Blood Genomic DNA Kit (Sigma Aldrich) as per the manufacturer's instructions. Following initial PCR amplification, allele-specific restriction enzyme digestion was performed and products analysed by electrophoresis on a 3% (w/v) agarose gel (Jiang et al., 1996).

Figure 4.2: Restriction digestion patterns

	Uncut	**R/R^{131} (GG)**	**H/R^{131} (AG)**	**H/H^{131} (AA)**
366bp	-			
343bp			-	-
322bp		-	-	



Genotype R/R R/R H/H H/R R/R R/R H/H H/R

4.3 Results

4.3.1 HLA region (rs3135394) and (rs9271366) genotyping results

Table 4.2: Association of HLA region (rs3135394) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	*OR*	*95% CI*
GG	0.3% (n=1)	4.9% (n=3)			
AG	15.3% (n=58)	37.7% (n=23)	****< 0.0001	0.276	0.168 to 0.451
AA	84.4% (n=320)	57.4% (n=35)			
	African controls (n=246)	African lupus nephritis (n=52)			
GG	0%	0%			
AG	1% (n=2)	2% (n=1)	0.439	0.420	0.0377 to 4.68
AA	99% (n=244)	98% (n=51)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
GG	0%	0%			
AG	0%	0%	-	-	-
AA	100% (n=197)	100% (n=13)			

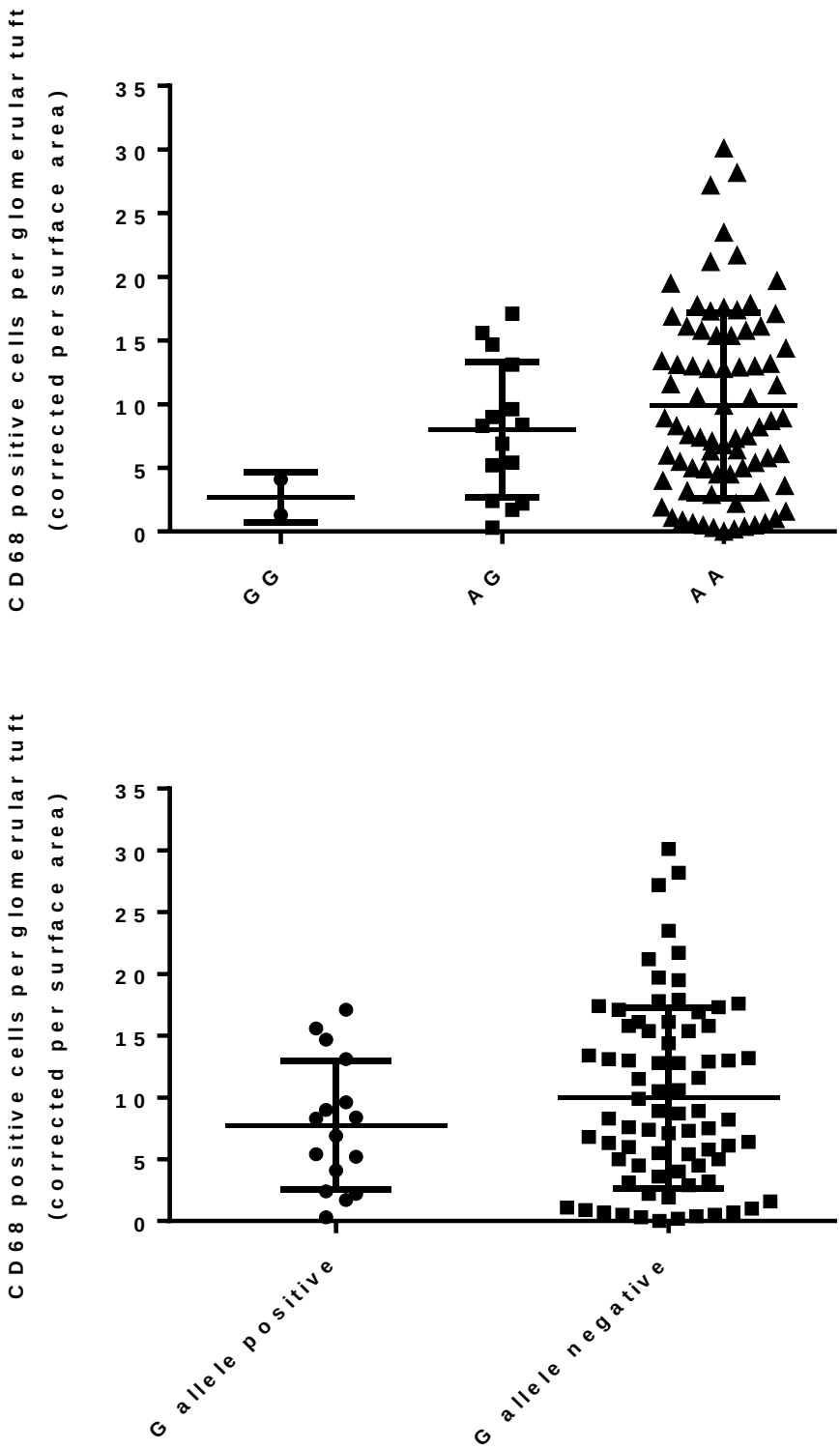
The difference in HLA-DRB1 (rs3135394) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.3: Univariate analysis exploring clinical parameters associated with the HLA region (rs3135394) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	2.6	0.575 to 11.8
First degree family history	0.12	0.00721 to 2.13
Juvenile onset (<18 years)	0.81	0.300 to 2.19
ESRD	0.70	0.150 to 3.31
ANA	1.47	0.173 to 12.5
Anti-dsDNA	0.80	0.331 to 1.95
Anti-Ro	1.3	0.549 to 3.08
Anti-RNP	0.44	0.165 to 1.18
Anti-Sm	0.48	0.133 to 1.71
APS	0.33	0.0732 to 1.48
Anti-cardiolipin	0.63	0.220 to 1.78
Lupus anticoagulant	0.34	0.111 to 1.05
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=28) Variable absent (n=132)	 28.0 ± 13.5 25.1 ± 10.6	0.543
Duration of follow-up (years) Variable present (n=28) Variable absent (n=132)	 12.9 ± 7.5 11.5 ± 7.3	0.324
Glomerular CD68 count Variable present (n=16) Variable absent (n=76)	 7.8 ± 5.2 10.0 ± 7.3	0.257

None statistically significant

Figure 4.3: CD68 immunostaining in HLA region (rs3135394) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft. There was no significant difference in CD68 positivity between GG, AG and AA genotypes nor was there a difference between those who were G allele positive or negative. Classes I, II and V excluded.

Table 4.4: Association of HLA region (rs9271366) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
GG	3% (n=13)	3% (n=2)			
AG	19% (n=73)	31% (n=21)	*0.029	0.583	0.835 to 1.00
AA	77% (n=293)	66% (n=38)			
	African controls (n=246)	African lupus nephritis (n=52)			
GG	3% (n=8)	3.8% (n=2)			
AG	27% (n=67)	44.2% (n=23)	*0.030	0.579	0.352 to 0.952
AA	70% (n=171)	51.9% (n=27)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
GG	2% (n=3)	0%			
AG	24% (n=48)	46.2% (n=6)	0.186	0.529	0.203 to 1.38
AA	74% (n=146)	53.8% (n=7)			

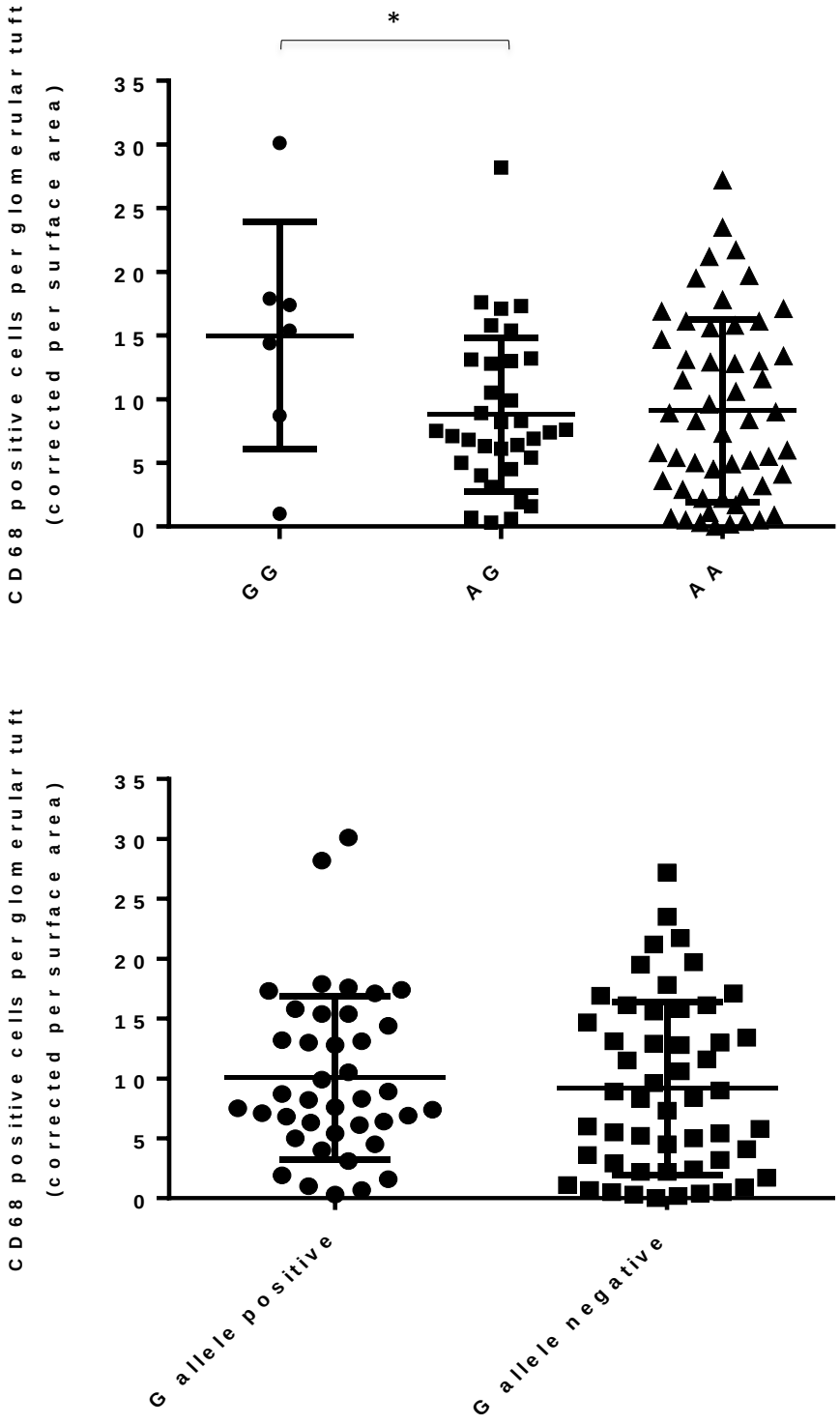
The difference in HLA-DRB1 (rs9271366) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.5: Univariate analysis exploring clinical parameters associated with the HLA region (rs9271366) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	0.64	0.267 to 1.53
First degree family history	2.70	0.928 to 7.84
Juvenile onset (<18 years)	1.80	0.890 to 3.65
ESRD	0.50	0.152 to 1.65
ANA	0.93	0.201 to 4.30
Anti-dsDNA	1.27	0.627 to 2.58
Anti-Ro	0.74	0.374 to 1.48
Anti-RNP	0.74	0.374 to 1.48
Anti-Sm	1.46	0.645 to 3.31
APS	2.05	0.888 to 4.73
Anti-cardiolipin	1.41	0.674 to 2.94
Lupus anticoagulant	1.48	0.741 to 2.97
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=65) Variable absent (n=95)	 24.9 ± 11.2 26.1 ± 11.2	0.645
Duration of follow-up (years) Variable present (n=65) Variable absent (n=95)	 11.2 ± 6.0 12.1 ± 8.2	0.905
Glomerular CD68 count Variable present (n=40) Variable absent (n=52)	 10.1 ± 6.8 9.2 ± 7.2	0.457

None statistically significant

Figure 4.4: CD68 immunostaining in HLA region (rs9271366) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). CD68 positivity was significantly higher in GG than AG genotypes (*p=0.028). There no difference between those who were G allele positive or negative. Classes I, II and V excluded.

4.3.2 Discussion of genotyping results of polymorphisms in the HLA region in lupus nephritis

The HLA region is consistently the most strongly associated region for genetic risk in SLE in GWAS and large replication studies. The HLA has proven challenging to study. It is the most gene-dense area of the genome and strong linkage disequilibrium (LD) spans the region making it difficult to ascertain if an identified variant is functional or in LD with functional polymorphisms nearby. The HLA is subdivided into 3 classes: I, II and III. SLE associations have been independently seen in Class II and Class III (Harley et al., 2008; Hom et al., 2008; Gateva et al., 2009). Multiple autoimmune and inflammatory diseases have been associated with polymorphisms in the HLA region including rheumatoid arthritis, type I diabetes, coeliac disease and Sjögren's syndrome.

We examined two of the most associated SNPs with SLE in the HLA region in our cohort and have shown an association between the rs3135394 risk allele and lupus nephritis in European patients and the rs9271366 risk allele and nephritis in European and African patients. Neither variant was associated with an increased rate of progression to ESRD.

Patients with the rs3135394 risk variant were more likely to have adult-onset sporadic disease. ANA and anti-Ro were more frequently seen in patients carrying the minor allele (OD 1.47, 95% CI 0.173 to 12.5 and OD 1.3, 95% CI .055 to 3.08 respectively).

Patients with the rs9271366 risk allele tended to be of younger onset. Familial nephritis was more frequently seen in those carrying the risk variant (OD 2.70, 95% CI 0.93 to 7.84). Anti-dsDNA and anti-Sm were more prevalent in those carrying the risk allele (OD 1.27, 95% CI 0.63 to 2.58 and OD 1.46, 95% CI 0.65 to 3.31

respectively). Antiphospholipid syndrome was more common in patients with the minor allele (OD 2.05, 95% CI 0.89 to 4.73). Anti-cardiolipin antibody and lupus anticoagulant were also more frequent in those with this risk variant.

Patients homozygous for the rs9271366 risk allele had a mean higher CD68 glomerular count than other genotypes. When CD68 was analysed comparing those who were positive or negative for the risk allele, however, no difference was seen.

Lupus nephritis patients with the rs3135394 risk variant were more likely to have lower CD68 glomerular counts but this did not reach statistical significance.

4.3.3 IRF5/IRF7/IRF8 genotyping results

IRF5 (rs4728142) and (rs2070197) genotyping results

Table 4.6: Association of IRF5 (rs4728142) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	20.6% (n=78)	21.3% (n=13)	0.741	0.938	0.639 to 1.38
AG	49.1% (n=186)	50.8% (n=31)			
GG	30.3% (n=115)	27.9% (n=17)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	4.5% (n=11)	11.5% (n=6)	0.066	0.649	0.408 to 1.03
AG	37.4% (n=92)	40.4% (n=21)			
GG	58.1% (n=143)	48.1% (n=25)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	0.5% (n=1)	0%	0.399	0.621	0.204 to 1.89
AG	19.3% (n=38)	30.8% (n=4)			
GG	80.2% (n=158)	69.2% (n=9)			

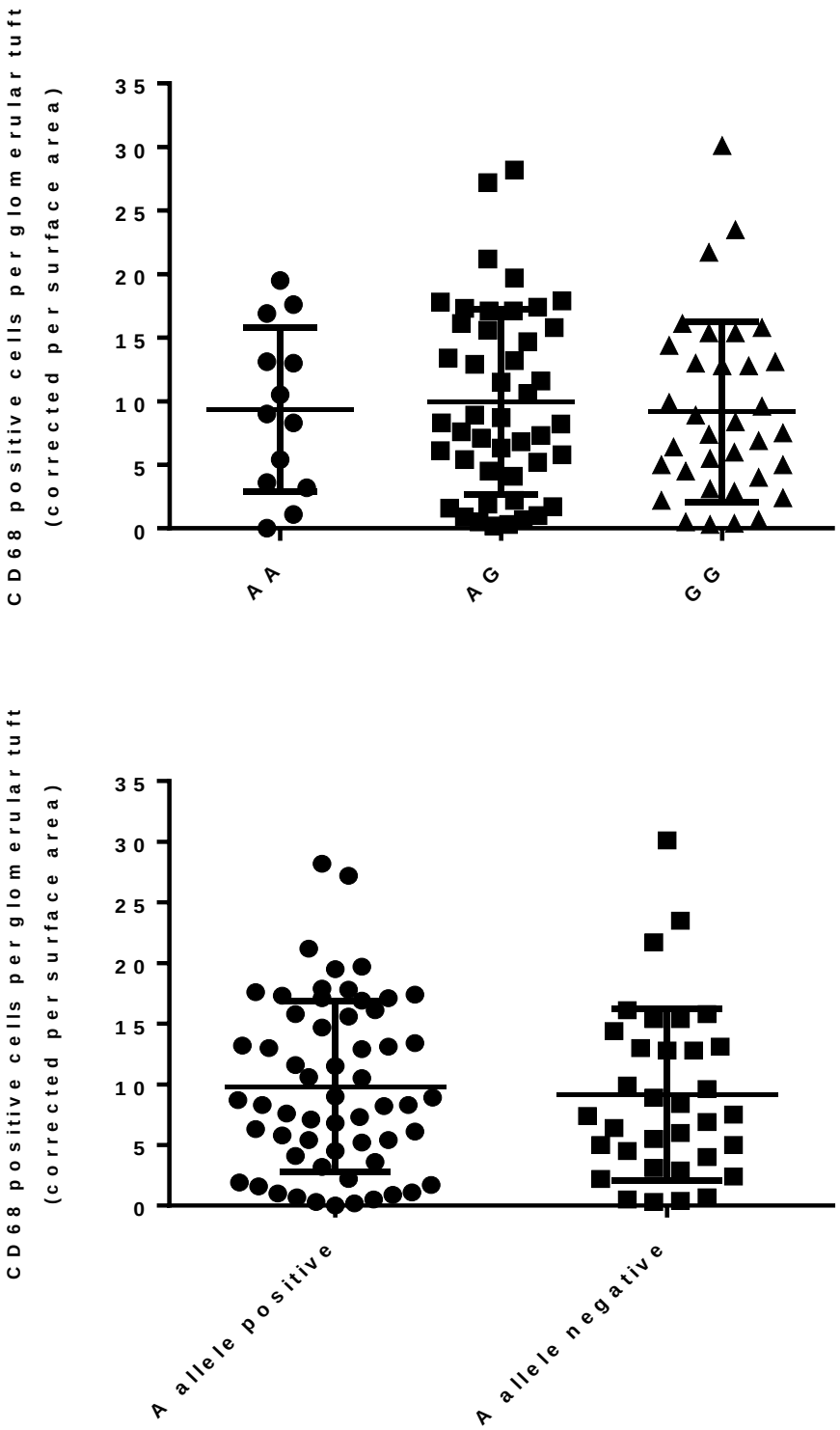
The difference in IRF5 (rs4728142) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.7: Univariate analysis exploring clinical parameters associated with the IRF5 (rs4728142) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	1.46	0.606 to 3.49
First degree family history	0.24**	0.0795 to 0.735
Juvenile onset (<18 years)	0.71	0.348 to 1.44
ESRD	2.67	0.721 to 9.87
ANA	0.62	0.117 to 3.32
Anti-dsDNA	0.63	0.303 to 1.32
Anti-Ro	1.02	0.515 to 2.03
Anti-RNP	0.88	0.443 to 1.75
Anti-Sm	1.47	0.617 to 3.51
APS	1.28	0.498 to 3.30
Anti-cardiolipin	1.05	0.439 to 2.51
Lupus anticoagulant	0.86	0.364 to 2.05
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=99) Variable absent (n=61)	 25.8 ± 10.5 25.2 ± 12.2	0.306
Duration of follow-up (years) Variable present (n=99) Variable absent (n=61)	 12.1 ± 7.3 11.1 ± 7.4	0.367
Glomerular CD68 count Variable present (n=58) Variable absent (n=34)	 9.8 ± 7.1 9.2 ± 7.1	0.547

***p*=0.0078, otherwise none statistically significant

Figure 4.5: CD68 immunostaining in IRF5 (rs4728142) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative. Classes I, II and V excluded.

Table 4.8: Association of IRF5 (rs2070197) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
GG	1.1% (n=4)	6.6% (n=4)	*0.019	0.536	0.317 to 0.907
AG	17.9% (n=68)	21.3% (n=13)			
AA	81.0% (n=307)	72.1% (n=44)			
	African controls (n=246)	African lupus nephritis (n=52)			
GG	0%	0%	0.075	0.276	0.0608 to 1.25
AG	1.6% (n=4)	5.8% (n=3)			
AA	98.4% (n=242)	94.2% (n=49)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
GG	0%	0%	-	-	-
AG	0.5% (n=1)	0%			
AA	99.5% (n=196)	100% (n=13)			

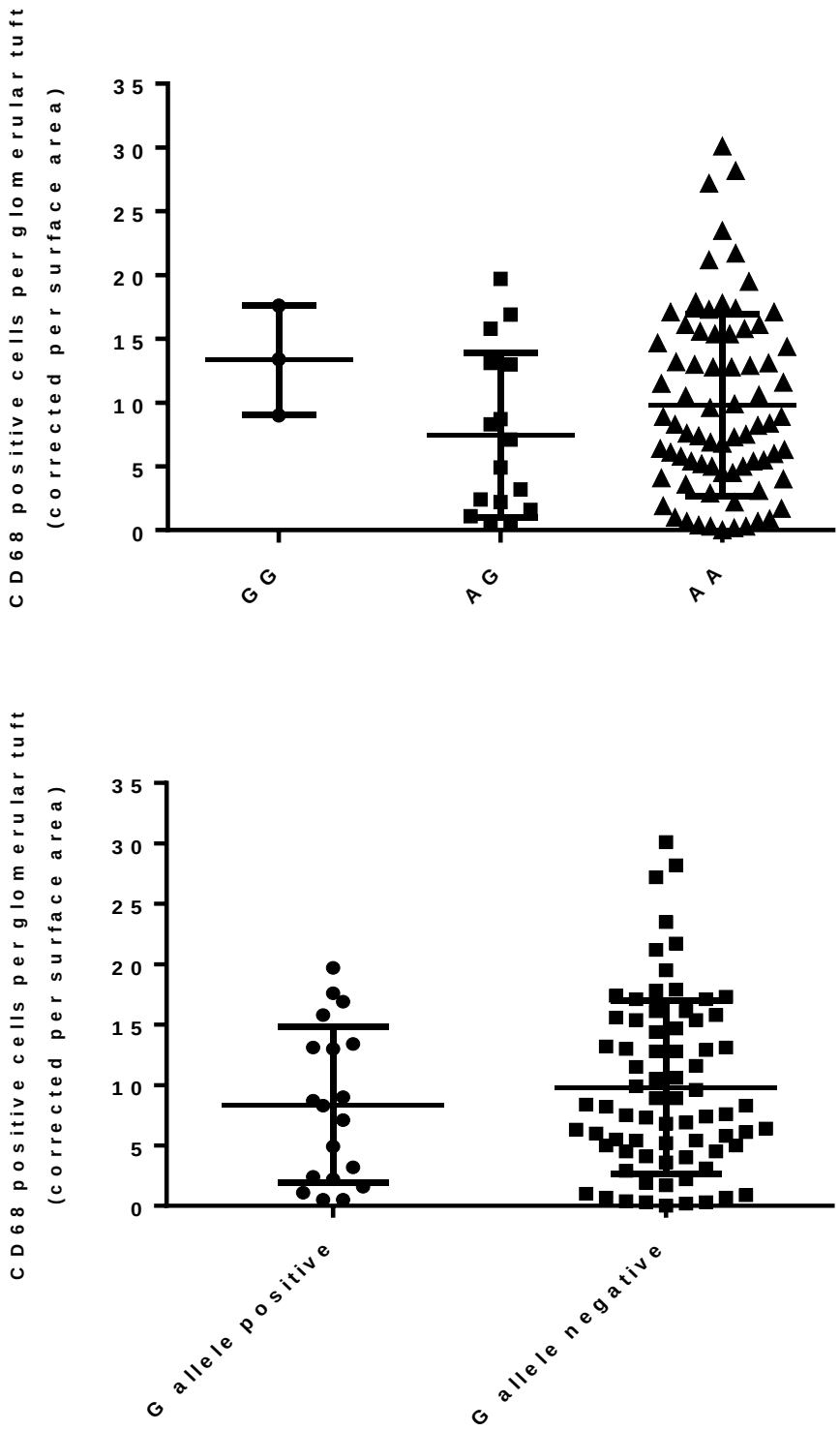
The difference in IRF5 (rs2070197) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.9: Univariate analysis exploring clinical parameters associated with the IRF5 (rs2070197) risk allele

Categorical Variables	**Odds ratio**	**95% Confidence Interval**
Gender (female)	0.86	0.292 to 2.51
First degree family history	0.26	0.0335 to 2.09
Juvenile onset (<18 years)	0.25*	0.0716 to 0.873
ESRD	0.12	0.00710 to 2.10
ANA	0.29	0.0614 to 1.37
Anti-dsDNA	0.66	0.287 to 1.54
Anti-Ro	1.12	0.480 to 2.60
Anti-RNP	0.50	0.197 to 1.27
Anti-Sm	0.63	0.201 to 1.99
APS	2.11	0.819 to 5.41
Anti-cardiolipin	1.53	0.627 to 3.74
Lupus anticoagulant	1.64	0.698 to 3.83
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=30) Variable absent (n=130)	 29.3 ± 11.4 24.9 ± 10.9	 *0.037
Duration of follow-up (years) Variable present (n=30) Variable absent (n=130)	 11.9 ± 6.8 11.7 ± 7.5	 0.669
Glomerular CD68 count Variable present (n=18) Variable absent (n=74)	 8.6 ± 6.6 9.8 ± 7.2	 0.673

*p=0.02, otherwise none statistically significant

Figure 4.6: CD68 immunostaining in IRF5 (rs2070197) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between GG, AG and AA genotypes nor was there a difference between those who were G allele positive or negative. Classes I, II and V excluded.

IRF7 (rs4963128) genotyping results

Table 4.10: Association of IRF7 (rs4963128) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	9% (n=34)	13.1% (n=8)	0.819	0.954	0.638 to 1.43
AG	48.8% (n=185)	42.6% (n=26)			
GG	42.2% (n=160)	44.3% (n=27)			
	African Controls (n=246)	African lupus nephritis (n=52)			
AA	22% (n=54)	11.5% (n=6)	0.112	1.42	0.920 to 2.18
AG	52% (n=128)	55.8% (n=29)			
GG	26% (n=64)	32.7% (n=17)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	1% (n=2)	0%	0.781	1.34	0.172 to 10.4
AG	8.1% (n=16)	7.7% (n=1)			
GG	90.9% (n=179)	92.3% (n=12)			

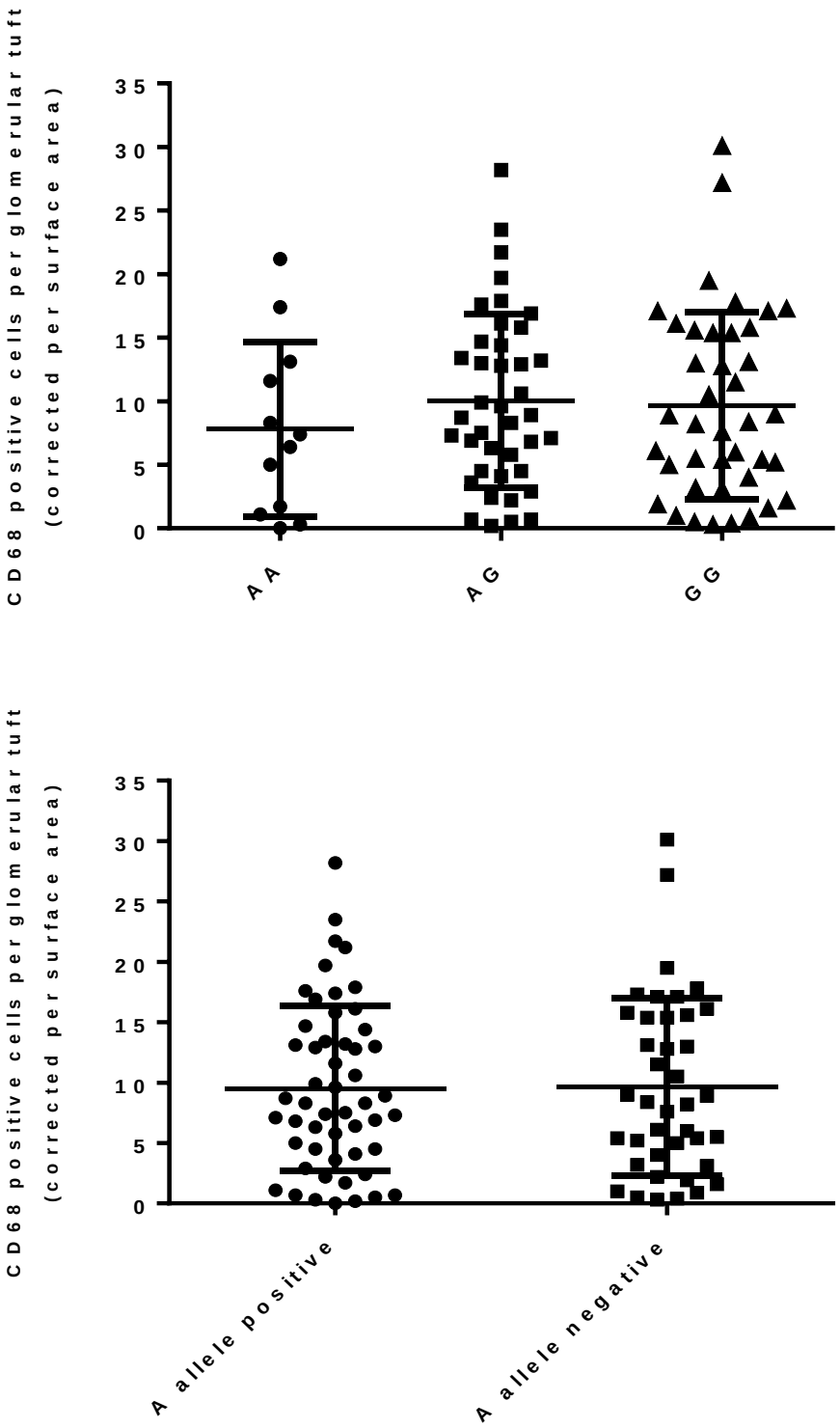
The difference in IRF7 (rs4963128) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.11: Univariate analysis exploring clinical parameters associated with the IRF7 (rs4963128) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	1.01	0.423 to 2.41
First degree family history	1.29	0.436 to 3.81
Juvenile onset (<18 years)	0.65	0.321 to 1.31
ESRD	0.71	0.245 to 2.06
ANA	1.58	0.343 to 7.33
Anti-dsDNA	1.60	0.798 to 3.19
Anti-Ro	1.29	0.656 to 2.54
Anti-RNP	1.89	0.950 to 3.78
Anti-Sm	1.66	0.711 to 3.87
APS	1.07	0.467 to 2.47
Anti-cardiolipin	1.08	0.517 to 2.25
Lupus anticoagulant	1.03	0.516 to 2.05
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=87) Variable absent (n=73)	 26.5 ± 11.3 24.6 ± 10.7	0.238
Duration of follow-up (years) Variable present (n=87) Variable absent (n=73)	 12.2 ± 7.9 11.1 ± 6.7	0.481
Glomerular CD68 count Variable present (n=52) Variable absent (n=40)	 9.5 ± 6.8 9.6 ± 7.3	0.945

None statistically significant

Figure 4.7: CD68 immunostaining in IRF7 (rs4963128) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative. Classes I, II and V excluded.

IRF8 (rs2280381) genotyping results

Table 4.12: Association of IRF8 (rs2280381) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
GG	13.5% (n=51)	6.6% (n=4)	0.574	1.12	0.751 to 1.68
AG	47.2% (n=179)	55.7% (n=34)			
AA	39.3% (n=149)	37.7% (n=23)			
	African Controls (n=246)	African lupus nephritis (n=52)			
GG	17.5% (n=43)	21.2% (n=11)	0.516	0.869	0.568 to 1.33
AG	50.4% (n=124)	50% (n=26)			
AA	32.1% (n=79)	28.8% (n=15)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
GG	1.5% (n=3)	0%	0.864	1.01	0.937 to 1.08
AG	22.3% (n=44)	23.1% (n=3)			
AA	76.1% (n=150)	76.9% (n=10)			

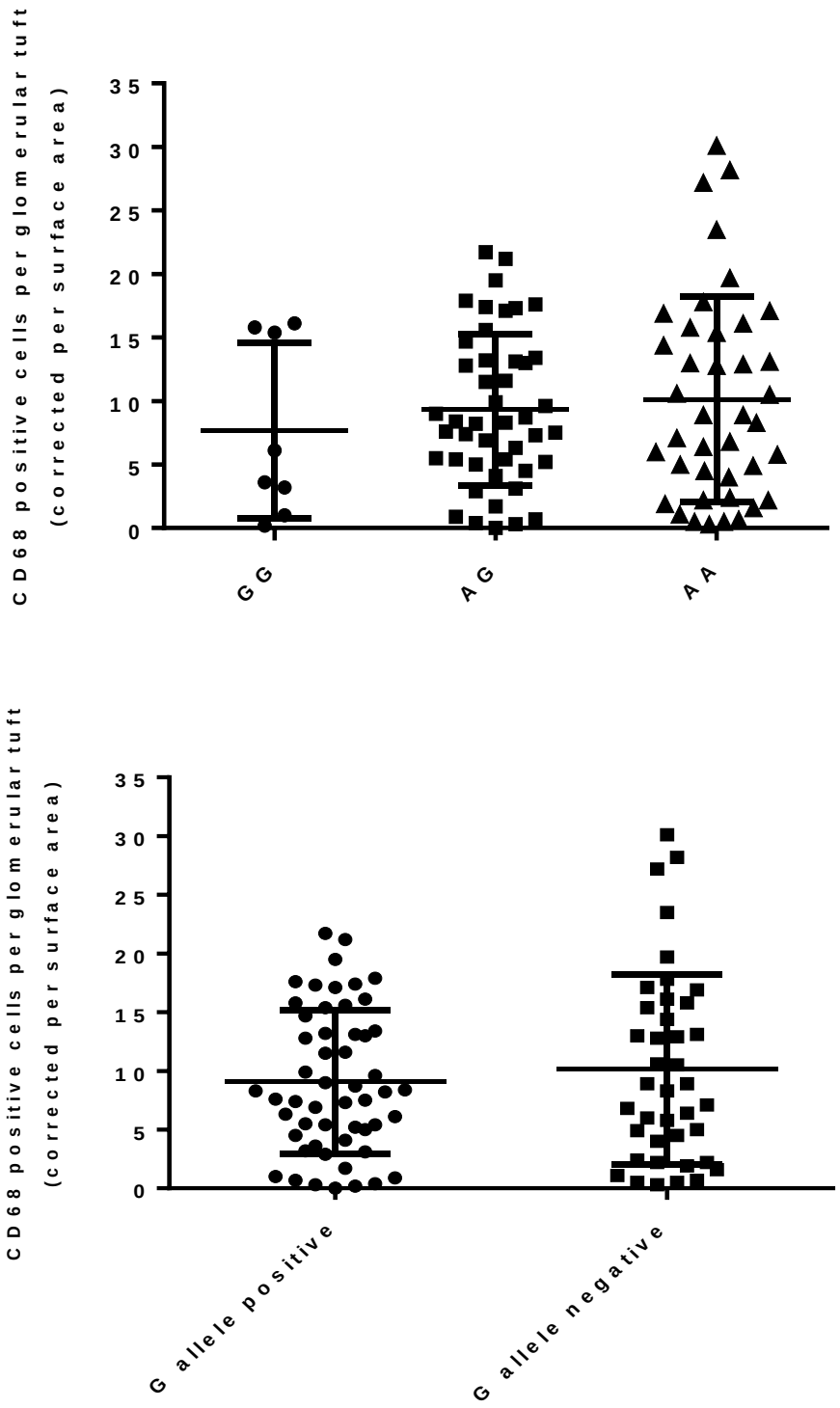
The difference in IRF8 (rs2280381) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.13: Univariate analysis exploring clinical parameters associated with the IRF8 (rs2280381) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	0.78	0.320 to 1.91
First degree family history	0.83	0.286 to 2.41
Juvenile onset (<18 years)	0.45	0.199 to 1.02
ESRD	0.83	0.286 to 2.41
ANA	0.42	0.0829 to 2.17
Anti-dsDNA	0.68	0.335 to 1.37
Anti-Ro	0.66	0.334 to 1.28
Anti-RNP	0.75	0.382 to 1.47
Anti-Sm	1.05	0.470 to 2.34
APS	0.54	0.233 to 1.24
Anti-cardiolipin	0.69	0.329 to 1.43
Lupus anticoagulant	0.67	0.333 to 1.33
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=92) Variable absent (n=68)	 26.1 ± 12.1 25.2 ± 10.1	0.922
Duration of follow-up (years) Variable present (n=92) Variable absent (n=68)	 10.6 ± 7.2 12.9 ± 7.4	*0.043
Glomerular CD68 count Variable present (n=53) Variable absent (n=39)	 9.1 ± 6.1 10.3 ± 8.2	0.752

None statistically significant

Figure 4.8: ***CD68 immunostaining in IRF8 (rs2280381) in proliferative lupus nephritis.***



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative. Classes I, II and V excluded.

4.3.4 Discussion of IRF5/IRF7/IRF8 genotyping results in lupus nephritis

One of the major defining features of SLE is increased type I interferon production with associated enhanced expression of interferon-inducible genes in PBMCs (Baechler et al., 2003; Bennett et al., 2003). It is not surprising, therefore, that a number of lupus susceptibility genes identified in GWAS and large scale replication studies are involved in interferon signalling pathways. These genes include *IRF5, IRF7, IRF8, STAT4, TYK2*, and *IFIH1*.

IRF5, IRF7 and *IRF8* are members of the interferon regulatory factor family (IRF) and play essential roles in the repertoire of cellular responses to interferons. Of these, *IRF5* has been the most extensively studied in the context of SLE to date. Variants in *IRF5* have been associated with SLE in multiple ancestral groups. Genome wide association with *IRF5* polymorphisms has been repeatedly seen in European and Han Chinese lupus patients (Harley et al, 2008; Hom et al, 2008; Han et al. 2009; (Lee et al., 2012). An association of *IFR5* polymorphisms has also been seen in Japanese, Mexican and African American SLE patients (Reddy et al., 2007; Kelly et al., 2008; Ito et al., 2009; Kawasaki et al., 2009; Lessard et al., 2012). *IRF5* SNPs have been associated with several other autoimmune and inflammatory diseases including Sjögren's syndrome, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease, (Dideberg et al., 2007; Miceli-Richard et al., 2007; Sigurdsson et al., 2007; Kristjansdottir et al., 2008).

Further evidence of the role of type I interferon in lupus pathogenesis is provided by the observation that *STAT4* and *IRF5* polymorphisms have been reported to act additively to increase lupus susceptibility (Abelson et al., 2009). Higher serum interferon-α activity has been demonstrated in SLE patients with the *IRF5* risk haplotype. Furthermore, the effect of the *IRF5* risk haplotype is more pronounced in

patients who are anti-dsDNA antibody or RNA-binding protein (anti-Ro, anti-Sm, anti-RNP) antibody positive (Niewold et al., 2008).

We examined two *IRF5* SNPs in lupus nephritis, rs4728142 and rs2070197, in our cohort. The *IRF5* rs4728142 risk allele was not associated with lupus nephritis in patients of any ancestry. The *IRF5* rs2070197 risk allele was associated with lupus nephritis in European patients in our study. There was a trend towards this variant being associated with lupus nephritis in African patients but this did not reach statistical significance.

Patients with lupus nephritis carrying the rs4728142 risk allele were more likely to progress to ESRD (OD 2.67, 95% CI 0.721 to 9.87). Both *IRF5* variants were more prevalent in sporadic onset disease than familial nephritis. Patients with the *IRF5* rs2070197 risk allele were significantly older at diagnosis of nephritis than those who did not carry the variant. Antiphospholipid syndrome was more prevalent in patients who carried the risk allele for either SNP.

Polymorphisms in *IRF7* have been associated with SLE in European and Han Chinese patients (Harley et al., 2008; Li et al., 2011). Subphenotype analysis in Chinese lupus patients has shown associations with lupus nephritis, arthritis and the production of anti-Ro and anti-Sm autoantibodies (Li et al., 2011).

In a study of European, Hispanic and African American SLE patients, the *IRF7* rs702966 risk allele was associated with the presence of anti-dsDNA antibodies. European American and Hispanic American patients who were homozygous for the rs702966 risk allele and anti-dsDNA antibody positive had higher serum levels of interferon-α. In African American patients, the *IRF7* rs4963128 SNP was associated

with anti-Sm antibodies and higher serum levels of interferon-α (Salloum et al., 2010).

We examined the *IRF7* rs4963128 polymorphism in our cohort but did not find any association with nephritis in any of the ancestral groups. Those carrying the risk allele had a higher prevalence of ANA and anti-dsDNA antibodies (OD 1.58, 95% CI 0.343 to 7.33) and (OD 1.60, 95% CI 0.798 to 3.10) respectively. Anti-Ro antibodies were more frequently positive in those who carried the risk variant (OD 1.29, 95% CI 0.656 to 2.54) as were anti-RNP and anti-Sm antibodies (OD 1.89, 95% CI 0.950 to 3.58 and OD 1.66, 95% CI 0.71 to 3.87, respectively).

Polymorphisms in *IRF8* were initially found to be associated with SLE in patients of European ancestry (Cunninghame-Graham et al., 2011). In a later study, an *IRF8* variant was found to have genome wide significance of association in a study of European, African American, Asian, Hispanic, Gullah, and Amerindian SLE patients (Lessard et al., 2012). Associations with *IRF8* polymorphisms have also been reported in multiple sclerosis patients (De Jager et al., 2009; Chrabot et al., 2013).

We did not see an association between the *IRF8* rs2280381 risk allele and lupus nephritis in our cohort of patients. There was no significant association of this SNP with autoantibody profile, disease onset or progression to ESRD.

No associations were seen regarding CD68 glomerular count in the different *IRF5/IRF7/IRF8* genotypes or in those with or without the risk allele.

4.3.5 ITGAM (rs1143679) (rs9888739) and (rs4548893) genotyping results.

Table 4.14: Association of ITGAM (rs1143679) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	0.8% (n=3)	8.2% (n=5)			
AG	25.1% (n=95)	31.1% (n=19)	**0.0025	0.493	0.309 to 0.786
GG	74.1% (n=281)	60.7% (n=37)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	1% (n=2)	5.8% (n=3)			
AG	22% (n=55)	28.8% (n=15)	*0.0258	0.539	0.310 to 0.934
GG	77% (n=189)	65.4% (n=34)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	0%	0%			
AG	0.5% (n=1)	0%	-	-	-
GG	99.5% (n=196)	100% (n=13)			

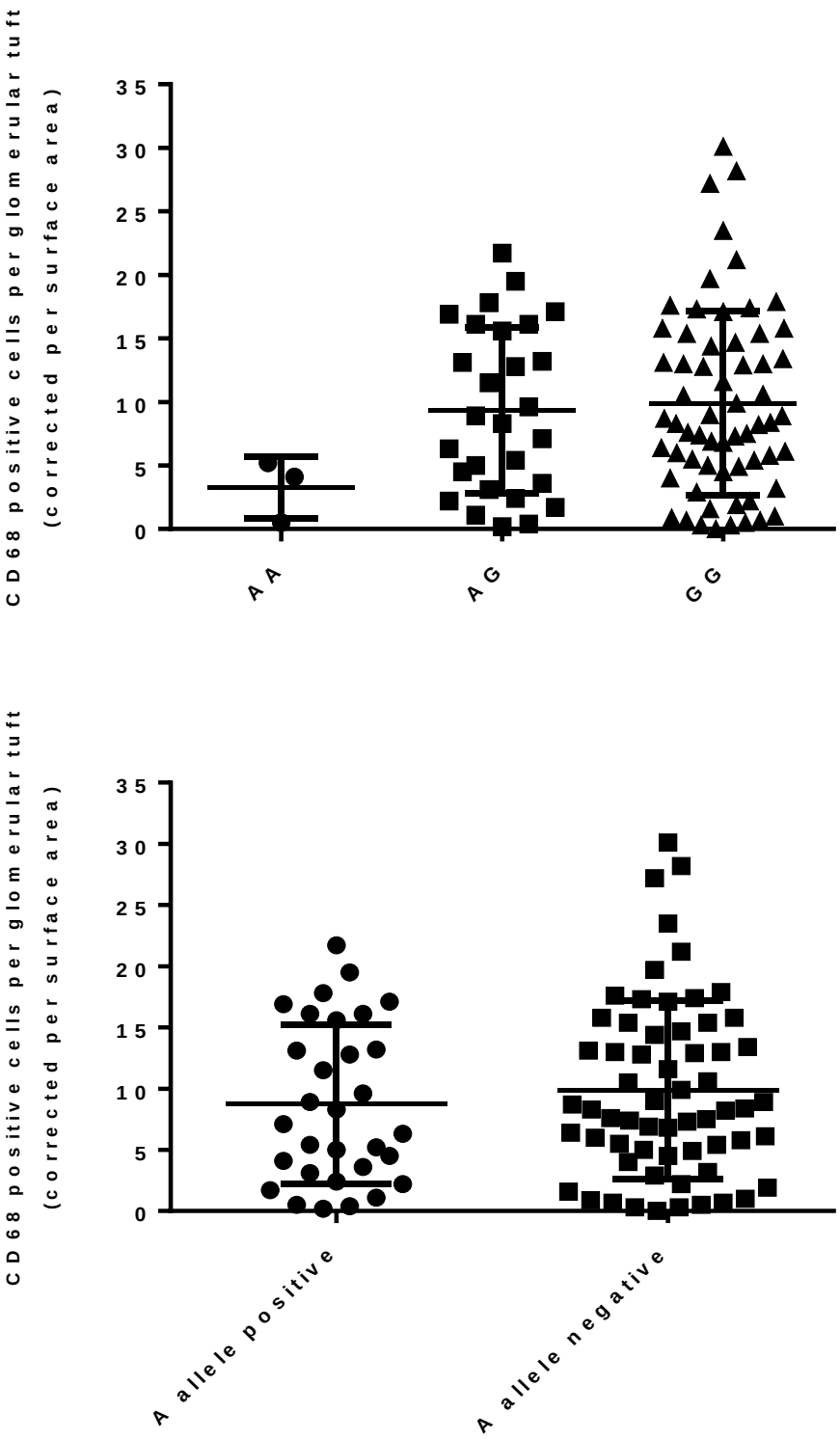
The difference in ITGAM (rs1143679) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.15: Univariate analysis exploring clinical parameters associated with the ITGAM (rs1143679) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	1.94	0.681 to 5.54
First degree family history	0.51	0.136 to 1.88
Juvenile onset (<18 years)	0.66	0.302 to 1.45
ESRD	0.46	0.125 to 1.70
ANA	5.2	0.282 to 96.2
Anti-dsDNA	0.80	0.387 to 1.67
Anti-Ro	0.72	0.344 to 1.49
Anti-RNP	0.75	0.358 to 1.55
Anti-Sm	1.15	0.488 to 2.72
APS	1.11	0.460 to 2.68
Anti-cardiolipin	1.14	0.524 to 2.47
Lupus anticoagulant	0.57	0.262 to 1.25
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=51) Variable absent (n=109)	 28.7 ± 12.6 24.1 ± 10.1	 *0.045
Duration of follow-up (years) Variable present (n=51) Variable absent (n=109)	 12.0 ± 7.0 11.5 ± 7.5	 0.493
Glomerular CD68 count Variable present (n=31) Variable absent (n=61)	 8.7 ± 6.5 10.0 ± 7.3	 0.504

None statistically significant

Figure 4.9: CD68 immunostaining in ITGAM (rs1143679) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative. Classes I, II and V excluded.

Table 4.16: Association of ITGAM (rs9888739) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	p-value	OD	95% CI
AA	1.3% (n=5)	10.6% (n=7)	**0.0021	0.504	0.323 to 0.785
AG	26.1% (n=99)	28.8% (n=19)			
GG	72.6% (n=275)	60.6% (n=40)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	43.1% (n=106)	36.5% (n=19)	0.914	1.02	0.660 to 1.59
AG	41.9% (n=103)	53.8% (n=28)			
GG	15.0% (n=37)	9.6% (n=5)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	0%	7.7% (n=1)	****< 0.0001	0.0305	0.00267 to 0.349
AG	0.5% (n=1)	0%			
GG	99.5% (n=196)	92.3% (n=12)			

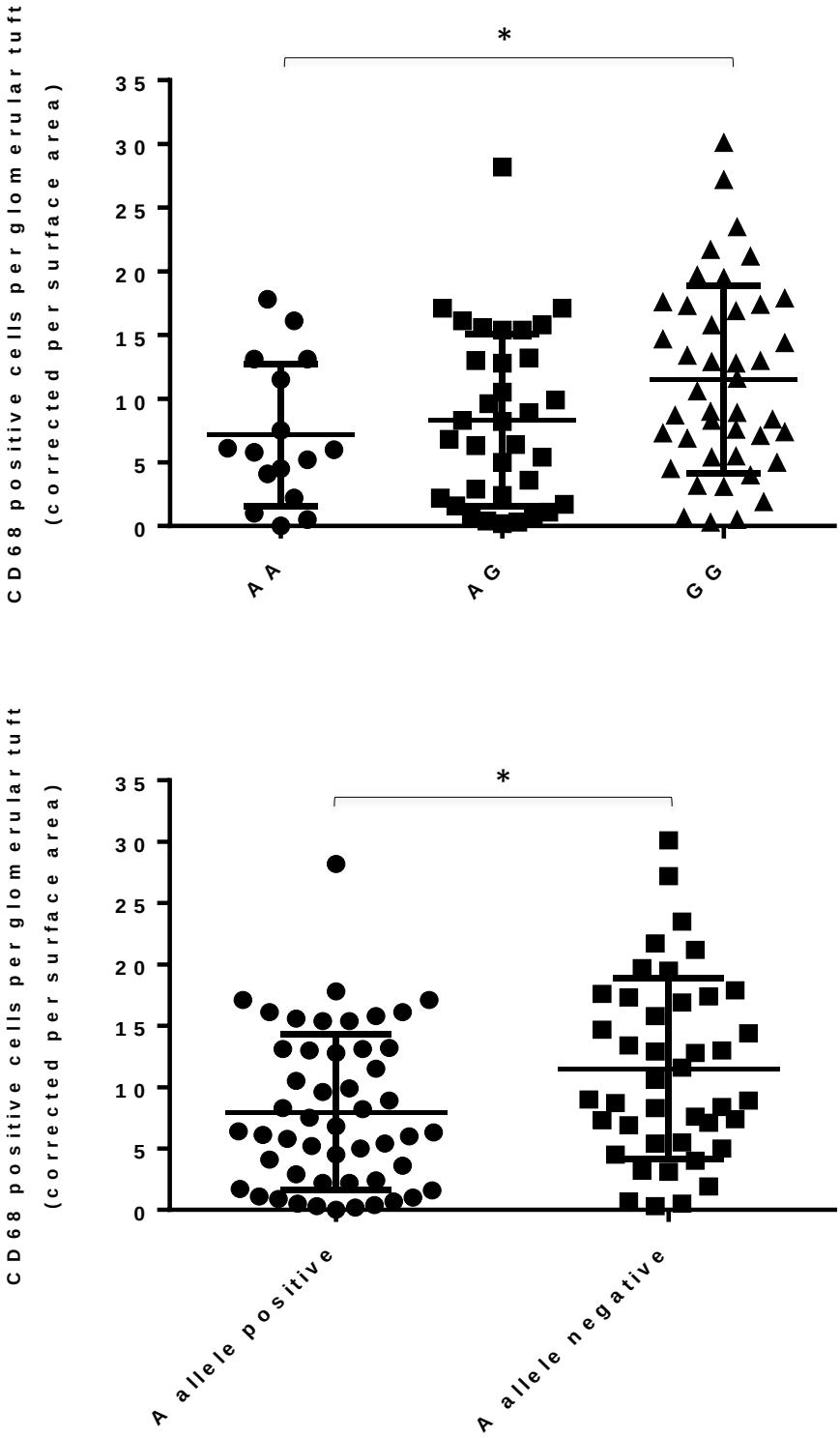
The difference in ITGAM (rs9888739) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

***Table 4.17: Univariate analysis exploring clinical parameters associated with the ITGAM (rs9888739) risk** allele*

Categorical Variables	**Odds ratio**	**95% Confidence Interval**
Gender (female)	0.92	0.387 to 2.21
First degree family history	1.04	0.357 to 3.01
Juvenile onset (<18 years)	0.63	0.313 to 1.28
ESRD	0.77	0.266 to 2.24
ANA	4.5	0.491 to 41.2
Anti-dsDNA	0.87	0.437 to 1.74
Anti-Ro	1.74	0.887 to 3.43
Anti-RNP	1.79	0.907 to 3.52
Anti-Sm	1.75	0.762 to 4.03
APS	0.98	0.429 to 2.25
Anti-cardiolipin	1.74	0.823 to 3.69
Lupus anticoagulant	0.96	0.483 to 1.91
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=84) Variable absent (n=76)	 27.6 ± 11.9 23.5 ± 9.8	 *0.02
Duration of follow-up (years) Variable present (n=84) Variable absent (n=76)	 11.1 ± 6.8 12.3 ± 7.8	 0.390
Glomerular CD68 count Variable present (n=) Variable absent (n=)	 7.9 ± 6.3 11.5 ± 7.4	 *0.02

None statistically significant

Figure 4.10: CD68 immunostaining in ITGAM (rs9888739) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). CD68 positivity was significantly lower in AA than GG genotypes (*p=0.038) and in those who were A allele positive than negative (*p=0.015). Classes I, II and V were excluded.

Table 4.18: Association of ITGAM (rs4548893) polymorphisms with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	4.2% (n=16)	11.5% (n=7)	*0.023	0.615	0.404 to 0.936
AG	35.1% (n=133)	39.3% (n=24)			
GG	60.7% (n=230)	49.2% (n=30)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	3.7% (n=9)	11.5% (n=6)	0.073	0.641	0.393 to 1.04
AG	30.9% (n=76)	30.7% (n=16)			
GG	65.4% (n=161)	57.7% (n=30)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	0%	0%	-	-	-
AG	0.5% (n=1)	0%			
GG	99.5% (n=196)	92.3% (n=13)			

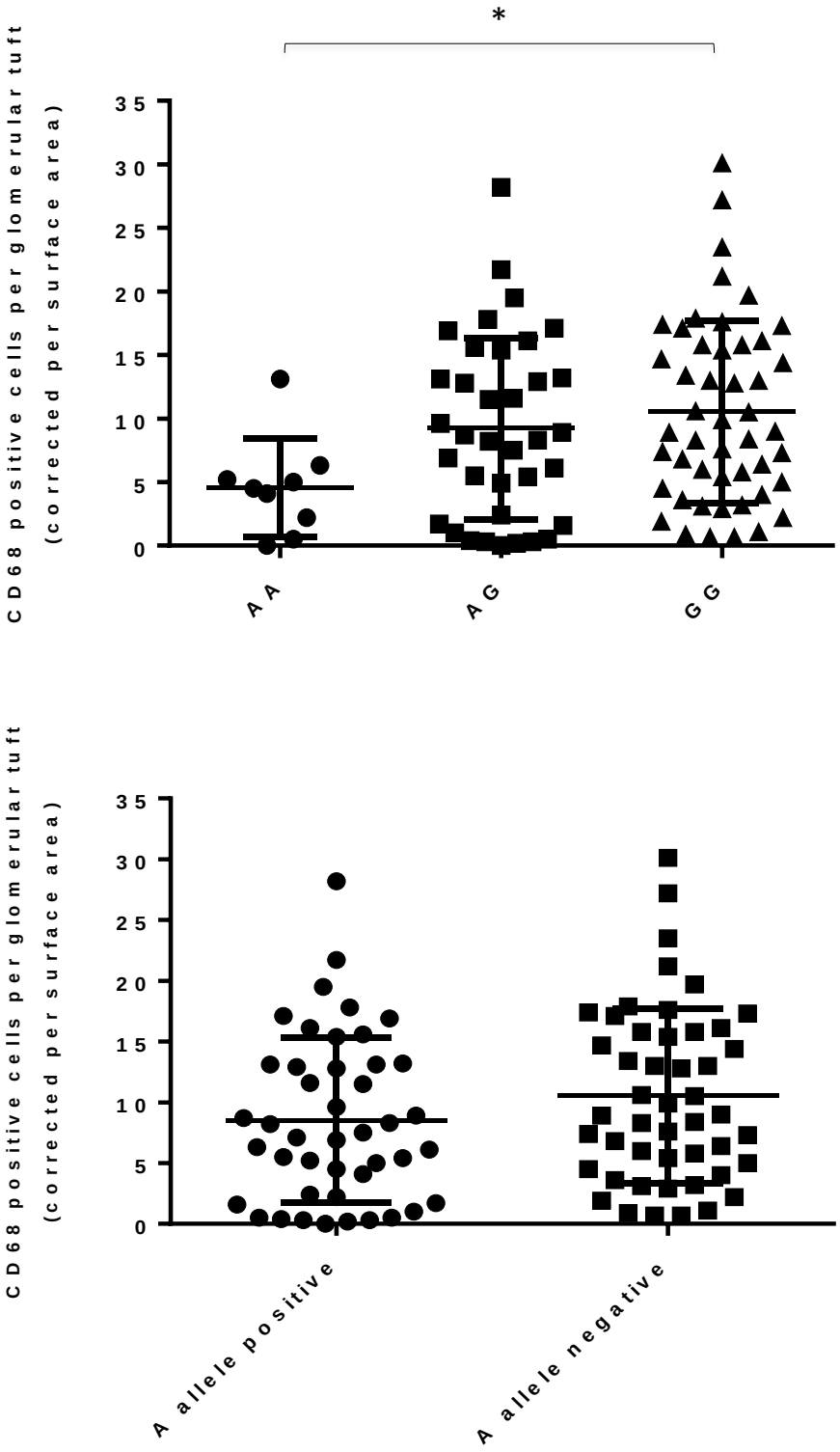
The difference in ITGAM (rs4548893) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.19: Univariate analysis exploring clinical parameters associated with the ITGAM (rs4548893) risk allele

Categorical Variables	**Odds ratio**	**95% Confidence Interval**
Gender (female)	0.72	0.302 to 1.72
First degree family history	0.45	0.136 to 1.47
Juvenile onset (<18 years)	1.06	0.524 to 2.15
ESRD	0.45	0.136 to 1.47
ANA	8.58	0.466 to 158
Anti-dsDNA	1.25	0.620 to 2.52
Anti-Ro	1.15	0.589 to 2.26
Anti-RNP	1.27	0.650 to 2.50
Anti-Sm	0.75	0.326 to 1.72
APS	1.86	0.808 to 4.30
Anti-cardiolipin	1.27	0.608 to 2.64
Lupus anticoagulant	1.70	0.849 to 3.40
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=69) Variable absent (n=91)	 26.7 ± 12.1 24.8 ± 10.4	0.299
Duration of follow-up (years) Variable present (n=69) Variable absent (n=91)	 12.0 ± 6.7 11.5 ± 7.8	0.366
Glomerular CD68 count Variable present (n=44) Variable absent (n=48)	 8.1 ± 6.7 10.1 ± 7.2	0.181

None statistically significant

Figure 4.11: CD68 immunostaining in ITGAM (rs4548893) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). CD68 positivity was significantly lower in AA than GG genotypes (*p=0.018). Classes I, II and V were excluded.

4.3.6 Discussion of ITGAM genotyping results in lupus nephritis

ITGAM encodes the integrin α_M protein, also known as CD11b which dimerizes with integrin β2 to form the cell surface receptor CR3 (complement receptor 3) also known as Mac-1. *ITGAM* is expressed on monocytes, macrophages, neutrophils and dendritic cells and plays important roles in leucocyte migration, adhesion and phagocytosis. Polymorphisms in *ITGAM* have been associated with SLE in European patients in several GWAS (Graham et al., 2008; Harley et al., 2008; Hom et al., 2008; Nath et al., 2008; Chung et al., 2011). Highest associations are consistently seen with the rs1143679 variant where an arginine/histidine amino acid substitution occurs at position 77 (R77H). The rs1143679 minor allele has been reported to impair phagocytosis and adhesion (MacPherson et al., 2011; Rhodes et al., 2012). Heterozygous 77R/H macrophages, monocytes and neutrophils have impaired phagocytosis with a reduced capacity to uptake complement coated targets as compared to 77R/R cells (Fossati-Jimack et al., 2013). *ITGAM* polymorphisms have also been associated with SLE in East Asian GWAS (Han et al., 2009; Yang et al., 2010; Fan et al., 2011). In some East Asian populations, however, only the non-risk allele of rs1143679 is present. The *ITGAM* rs1143679 risk allele has been associated specifically with lupus nephritis and also with discoid skin features (Yang et al., 2009; Kim-Howard et al., 2010).

We examined 3 *ITGAM* polymorphisms, rs1143679, rs9888739 and rs4548893, in our study. The rs1143679 risk allele was associated with lupus nephritis in European and African patients. No East Asian patient carried the risk allele. ANA positivity was more prevalent in those carrying the rs1143679 risk variant (OD 5.2, 95% CI 0.282 to 96.2).

The *ITGAM* rs9888739 polymorphism was associated with lupus nephritis in European and East Asian patients. Those carrying the risk allele had higher frequency of ANA and anti-cardiolipin positivity (OD 4.5, 05% CI 0.491 to 41.2 and OD 1.74, 95% CI 0.823 to 3.69, respectively). Anti-Ro antibodies were more frequently positive in those with the risk variant (OD 1.74, 95% CI 0.887 to 3.43), as were anti-RNP and anti-Sm antibodies (OD 1.79, 95% CI 0.907 to 3.52 and OD 1.75, 95% CI 0.762 to 4.03, respectively).

The *ITGAM* rs4548893 risk variant was associated with lupus nephritis in European patients and there was a trend for an association in African patients. ANA and anti-dsDNA antibodies were more likely to be positive in those carrying the risk allele (OD 8.58, 95% CI 0.466 to 158 and OD 1.25, 95% CI 0.620 to 2.52, respectively). Anti-Ro and anti-RNP antibodies were more frequently positive in lupus nephritis patients with the rs4548893 minor allele (OD 1.15, 95% CI 0.589 to 2.26 and OD 1.27, 95% CI 0.650 to 2.50, respectively). Antiphospholipid syndrome was more common in individuals with the risk variant (OD 1.86, 95% CI 0.808 to 4.30) as were anti-cardiolipin antibody and lupus anticoagulant.

Lupus nephritis patients carrying the *ITGAM* rs1143679 or rs9888739 risk variants were significantly older at disease diagnosis than those without the risk alleles. There was no increased frequency of familial nephritis or progression to ESRD associated with any of the *ITGAM* risk alleles. Glomerular CD68 count was lower in those homozygous for the risk allele for all 3 polymorphisms. Three female nephritis patients of European ancestry were homozygous for all 3 *ITGAM* polymorphisms and had the lowest CD68 glomerular score.

4.3.7 STAT4 (rs7574865) and (rs3821236) genotyping results

Table 4.20: Association of STAT4 (rs7574865) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	5.5% (n=21)	13.1% (n=8)	***0.0005	2.03	1.36 to 3.04
AG	34.8% (n=132)	49.1% (n=30)			
GG	59.6% (n=226)	37.7% (n=23)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	3.7% (n=9)	11.5% (n=6)	**0.0037	2.11	1.27 to 3.53
AG	19.9% (n=49)	26.9% (n=14)			
GG	76.4% (n=188)	61.5% (n=32)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	8.1% (n=16)	7.7% (n=1)	0.735	0.862	0.365 to 2.04
AG	54.5% (n=102)	46.2% (n=6)			
GG	40.1% (n=79)	46.2% (n=6)			

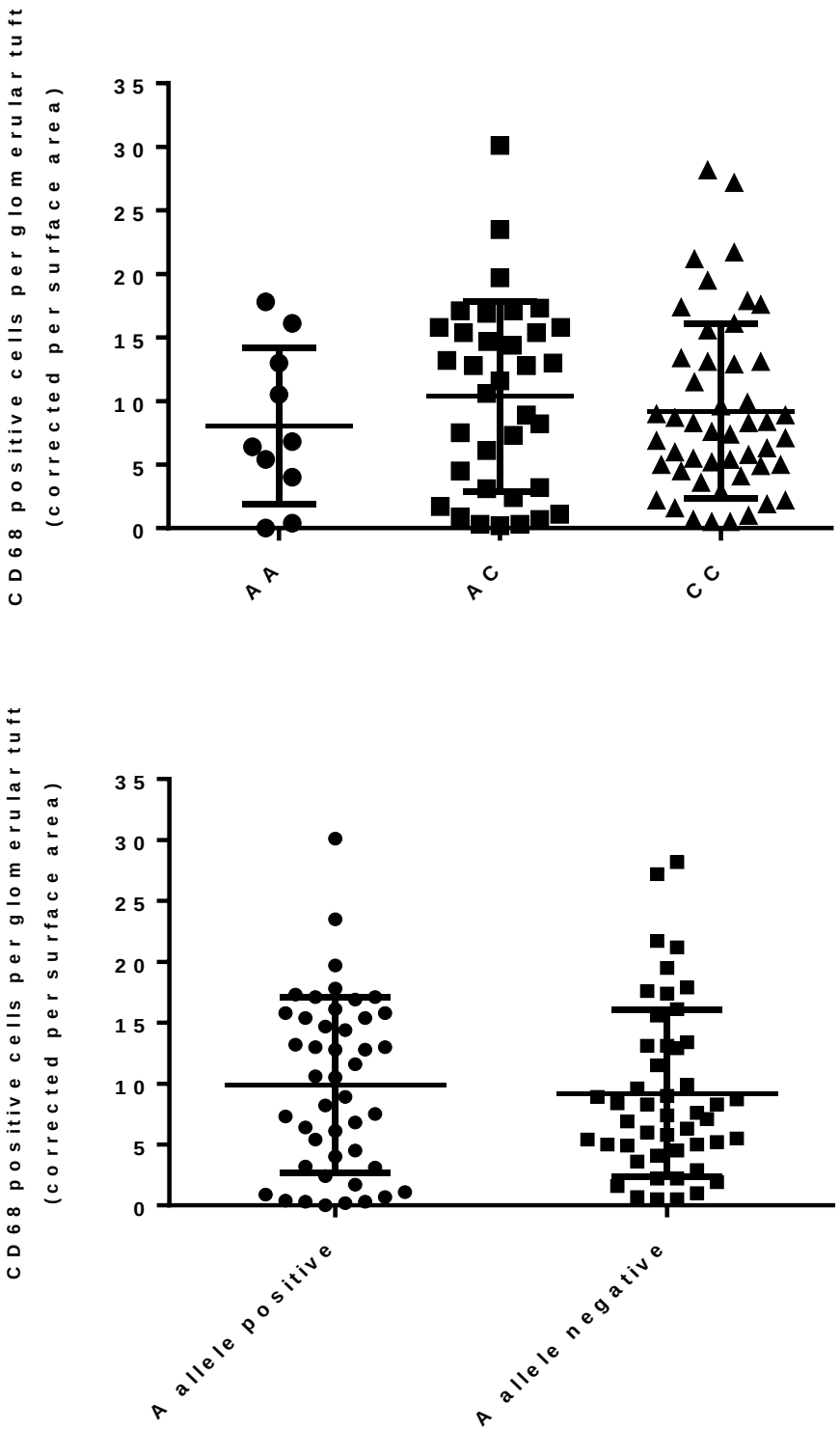
The difference in STAT4 (rs7574865) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.21: Univariate analysis exploring clinical parameters associated with the STAT4 (rs7574865) risk allele.

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	0.75	0.315 to 1.80
First degree family history	0.51	0.165 to 1.56
Juvenile onset (<18 years)	1.53	0.755 to 3.08
ESRD	0.94	0.323 to 2.72
ANA	0.68	0.148 to 3.16
Anti-dsDNA	1.30	0.650 to 2.60
Anti-Ro	0.55	0.279 to 1.08
Anti-RNP	0.71	0.362 to 1.39
Anti-Sm	0.81	0.359 to 1.84
APS	0.85	0.370 to 1.95
Anti-cardiolipin	1.07	0.515 to 2.22
Lupus anticoagulant	0.84	0.421 to 1.68
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=77) Variable absent (n=83)	 23.8 ± 10.7 27.3 ± 11.3	 *0.041
Duration of follow-up (years) Variable present (n=77) Variable absent (n=83)	 12.3 ± 7.1 11.2 ± 7.6	 0.189
Glomerular CD68 count Variable present (n=45) Variable absent (n=47)	 9.9 ± 7.2 9.3 ± 6.9	 0.769

None statistically significant

Figure 4.12: CD68 immunostaining in STAT4 (rs7574865) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AC and CC genotypes nor was there a difference between those who were A allele positive or negative.

Table 4.22: Association of STAT4 (rs3821236) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)		OD	95% CI
AA	2.6% (n=10)	6.6% (n=4)	***0.0002	0.459	0.303 to 0.697
AG	32.5% (n=123)	54.1% (n=33)			
GG	64.9% (n=246)	39.3% (n=24)			
	African Controls (n=246)	African lupus nephritis (n=52)			
AA	9.3% (n=23)	21.2% (n=11)	0.061	0.660	0.427 to 1.02
AG	43.1% (n=106)	38.5% (n=20)			
GG	47.6% (n=117)	40.4% (n=21)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	17.8% (n=35)	16.7% (n=3)	0.614	0.815	0.367 to 1.81
AG	46.7% (n=92)	50% (n=6)			
GG	35.5% (n=70)	33.3% (n=4)			

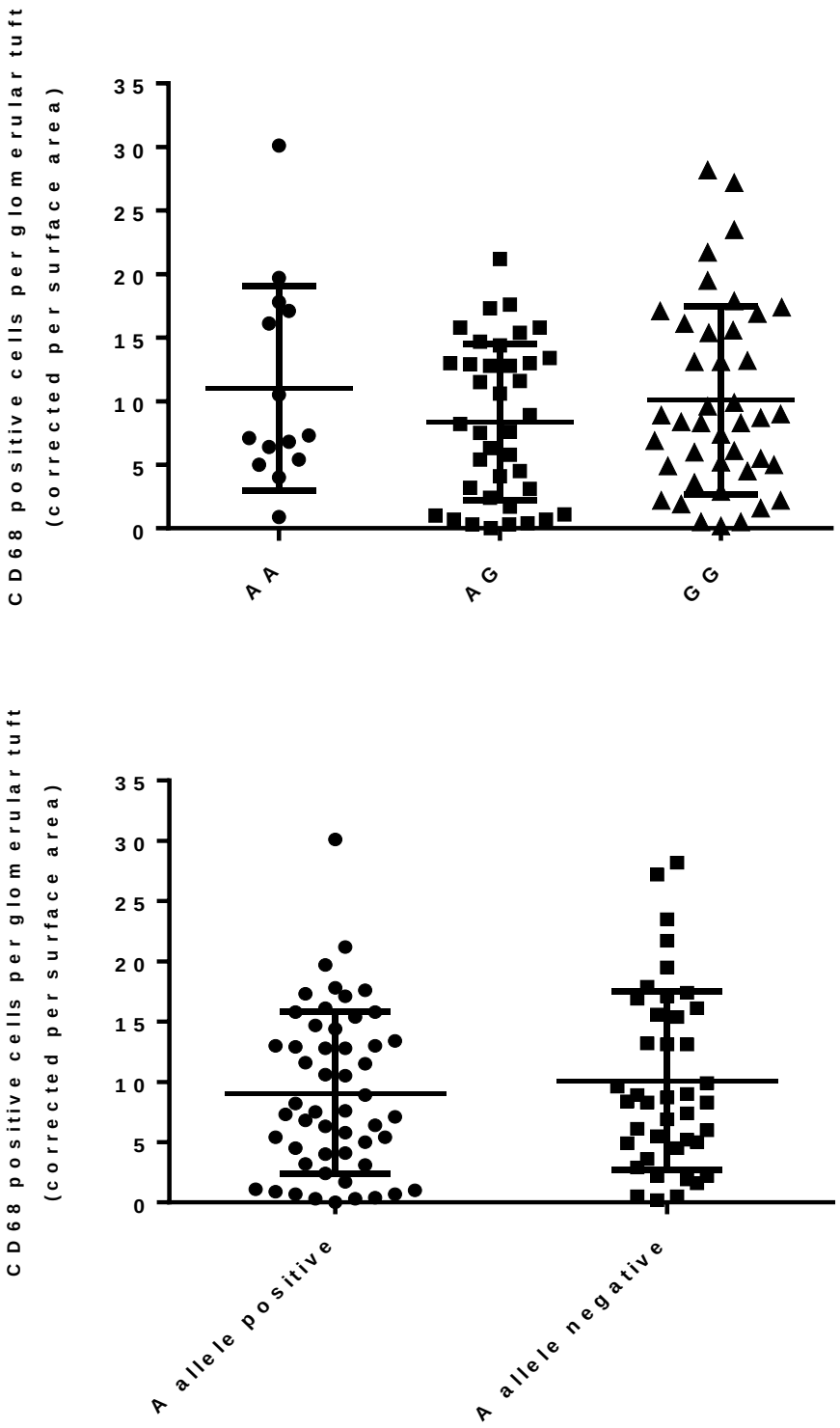
The difference in STAT4 (rs3821236) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.23: Univariate analysis exploring clinical parameters associated with the STAT4 (rs3821236) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	1.14	0.476 to 2.72
First degree family history	0.85	0.294 to 2.48
Juvenile onset (<18 years)	1.60	0.775 to 3.30
ESRD	1.58	0.514 to 4.86
ANA	0.99	0.215 to 4.59
Anti-dsDNA	1.17	0.584 to 2.34
Anti-Ro	0.66	0.334 to 1.28
Anti-RNP	0.75	0.382 to 1.47
Anti-Sm	1.29	0.560 to 2.97
APS	0.77	0.335 to 1.77
Anti-cardiolipin	0.56	0.265 to 1.16
Lupus anticoagulant	0.55	0.272 to 1.09
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=91) Variable absent (n=69)	 24.6 ± 10.6 27.9 ± 11.8	 0.197
Duration of follow-up (years) Variable present (n=91) Variable absent (n=69)	 11.9 ± 6.9 11.3 ± 7.9	 0.336
Glomerular CD68 count Variable present (n=52) Variable absent (n=40)	 9.1 ± 6.7 10.2 ± 7.4	 0.432

None statistically significant

Figure 4.13: CD68 immunostaining in STAT4 (rs3821236) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative.

4.3.8 Discussion of STAT4 genotyping results in lupus nephritis

STAT4 (signal transducer and activator of transcription 4) is a member of the STAT family of transcription factors. In addition to its role in type I interferon signaling, *STAT4* is involved in Th1 and Th17 differentiation and Il-17 and interferon-γ production (Nguyen et al., 2002; Watford et al., 2004; Mathur et al., 2007). SLE patients with the *STAT4* rs7574865 risk variant have increased expression of type I interferon regulated genes (Kariuki et al., 2009).

STAT4 polymorphisms have been associated with SLE in European, East Asian and African patients (Chung et al., 2009; Han et al., 2009; Namjou et al., 2009). *STAT4* variants have also been associated with rheumatoid arthritis and antiphospholipid syndrome (Remmers et al., 2007; Horita et al., 2009; Yin et al., 2009). *STAT4* risk variants have been associated with a more severe clinical phenotype of SLE. Taylor et al reported that the rs7574825 risk allele was associated with anti-dsDNA antibodies, lupus nephritis and younger disease onset. Patients with ESRD had an even higher association with the risk variant in their study (Taylor et al., 2008). A further study showed an association between the *STAT4* rs7582694 polymorphism and severe renal insufficiency (GRF<30mL/min) in proliferative lupus nephritis (Bolin et al., 2013).

We examined two *STAT4* polymorphisms previously associated with SLE, rs7574865 and rs3821236, in our cohort. The *STAT4* rs7574865 risk allele was associated with lupus nephritis in European and African patients in our study. Carriers of the risk variant did not have an increased progression to ESRD but they did have significantly younger onset of nephritis than those without the risk allele.

Anti-dsDNA antibodies were more prevalent in patients with the risk variant (OD 1.30, 95% CI 0.650 to 2.60).

The *STAT*4 rs3821236 risk allele was associated with lupus nephritis in European patients in our study. A trend was seen in African nephritis patients but this did not reach statistical significance. Juvenile onset nephritis was more frequent in the risk allele carrying patients (OD 1.60, 95% CI 0.775 to 3.30). Patients with the risk variant were more likely to progress to ESRD (OD 1.58, 95% CI 0.514 to 4.86). Anti-dsDNA and anti-Sm antibodies were more prevalent in lupus nephritis patients with the risk allele (OD 1.17, 95% CI 0.584 to 2.34 and OD 1.29, 95% CI 0.560 to 2.97, respectively).

4.3.9 IKZF1 (rs4917014) genotyping results

Table 4.24: Association of IKZF1 (rs4917014) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
CC	10.3% (n=39)	1.6% (n=1)	**0.0013	2.08	1.32 to 3.28
AC	47.5% (n=180)	39.3% (n=24)			
AA	42.2% (n=160)	59.1% (n=36)			
	African controls (n=246)	African lupus nephritis (n=52)			
CC	0.4% (n=1)	1.9% (n=1)	0.954	0.971	0.360 to 2.62
AC	8.5% (n=21)	5.8% (n=3)			
AA	91.1% (n=224)	92.3% (n=48)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
CC	12.2% (n=24)	7.7% (n=1)	0.074	2.42	0.893 to 6.56
AC	48.7% (n=96)	23.1% (n=3)			
AA	39.1% (n=77)	69.2% (n=9)			

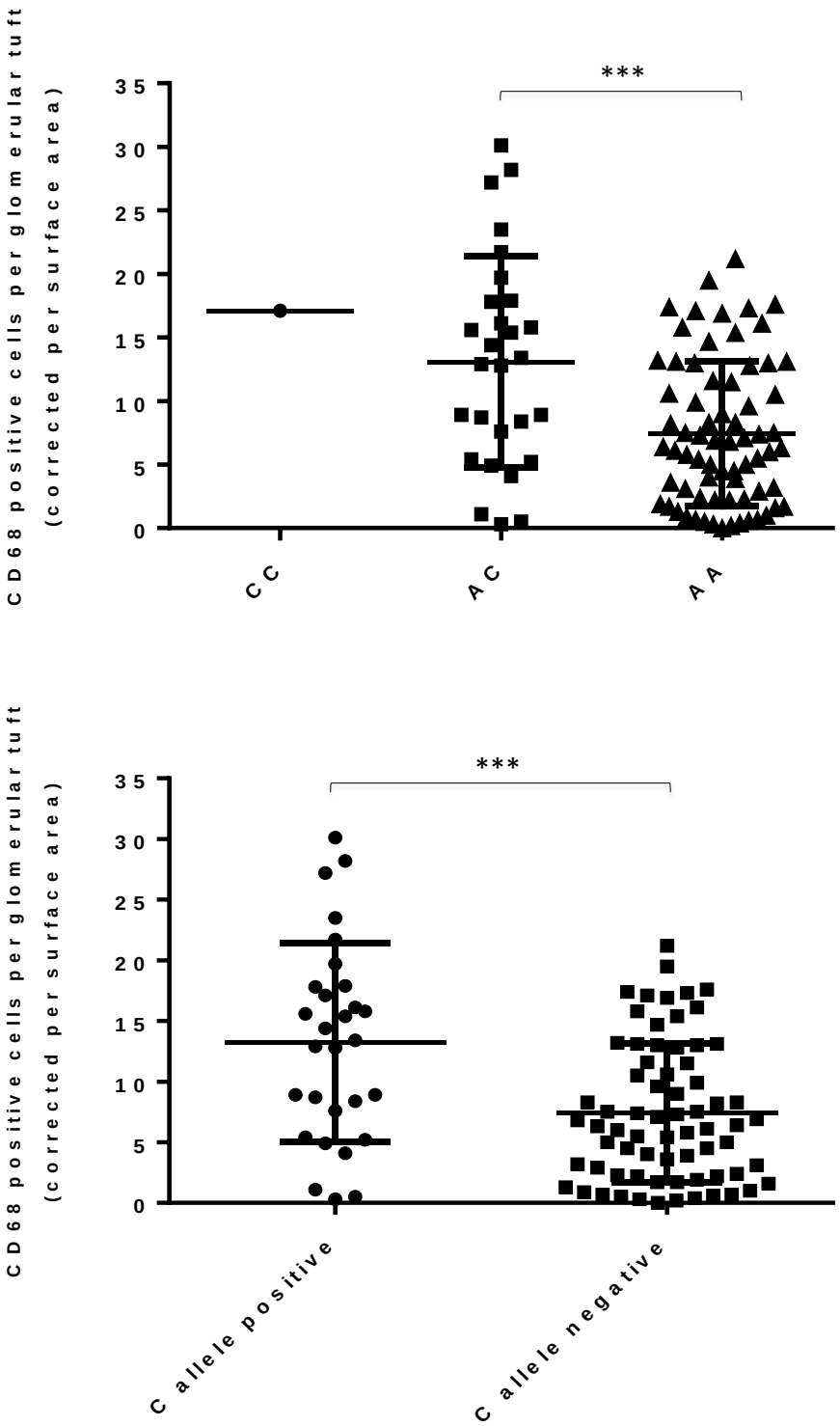
The difference in IKZF1 (rs4917014) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.25: Univariate analysis exploring clinical parameters associated with the IKZF1 (rs4917014) risk allele

Categorical Variables	**Odds ratio**	**95% Confidence Interval**
Gender (female)	0.58	0.239 to 1.42
First degree family history	0.52	0.140 to 1.94
Juvenile onset (<18 years)	1.66	0.800 to 3.46
ESRD	0.78	0.236 to 2.59
ANA	0.58	0.126 to 2.72
Anti-dsDNA	0.77	0.369 to 1.60
Anti-Ro	0.51	0.242 to 1.09
Anti-RNP	0.60	0.272 to 1.33
Anti-Sm	0.36	0.129 to 1.02
APS	0.90	0.365 to 2.23
Anti-cardiolipin	0.89	0.400 to 1.99
Lupus anticoagulant	1.13	0.539 to 2.36
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=50) Variable absent (n=110)	 23.2 ± 11.3 26.7 ± 11.0	 *0.048
Duration of follow-up (years) Variable present (n=50) Variable absent (n=110)	 11.5 ± 6.5 11.8 ± 7.7	 0.926
Glomerular CD68 count Variable present (n=28) Variable absent (n=64)	 13.5 ± 8.2 7.8 ± 5.7	 **0.0013

None statistically significant

Figure 4.14: CD68 immunostaining in IKZF1 (rs4917014) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). CD68 positivity significantly higher in AC than AA genotype (****p*=0.0002), otherwise there was no difference between genotypes. CD68 was significantly higher in C allele positive individuals (****p*= 0.0001).

4.3.10 Discussion of IKZF1 genotyping results in lupus nephritis

Ikaros proteins are zinc finger DNA-binding transcription factors that are essential regulators of lymphocyte differentiation (Klug et al., 1998; Harker et al., 2002). *IKZF1* (Ikaros family zinc-finger1) plays a role in regulation of *STAT4* transcription (Yap et al., 2005). Polymorphisms in *IKZF1* have been associated with SLE in European and Han Chinese patients (Han et al, 2009; He et al., 2010; Cunninghame-Graham et al., 2011). A subphenotype analysis in East Asian SLE patients found associations between the *IKZF1* rs4917014 risk variant with malar rash and renal nephritis (He et al., 2010)

The *IKZF1* rs4917014 risk allele was not associated with lupus nephritis in our cohort. The risk allele was more prevalent in the 1,000 Genomes Project European control population than in our European patients in fact. Patients with the risk allele were significantly younger at disease onset than those without the variant, however.

Renal biopsy tissue was only available for one patient who was homozygous for the risk allele. CD68 glomerular immunostaining was significantly higher in those who carried the risk variant as compared to those who did not (***p= 0.0001).

4.3.11 IFIH1 (rs1990760) genotyping results

Table 4.26: Association of IFIH1 (rs1990760) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	35.4% (n=134)	47.5% (n=29)	0.229	0.781	0.522 to 1.17
AG	50.6% (n=192)	37.7% (n=23)			
GG	14% (n=53)	14.8% (n=9)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	2% (n=5)	3.8% (n=2)	0.417	1.36	0.648 to 2.84
AG	18.7% (n=46)	9.6% (n=5)			
GG	79.3% (n=195)	86.5% (n=45)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	3% (n=6)	0%	0.613	0.784	0.304 to 2.02
AG	32% (n=63)	46.2% (n=6)			
GG	65% (n=128)	53.8% (n=7)			

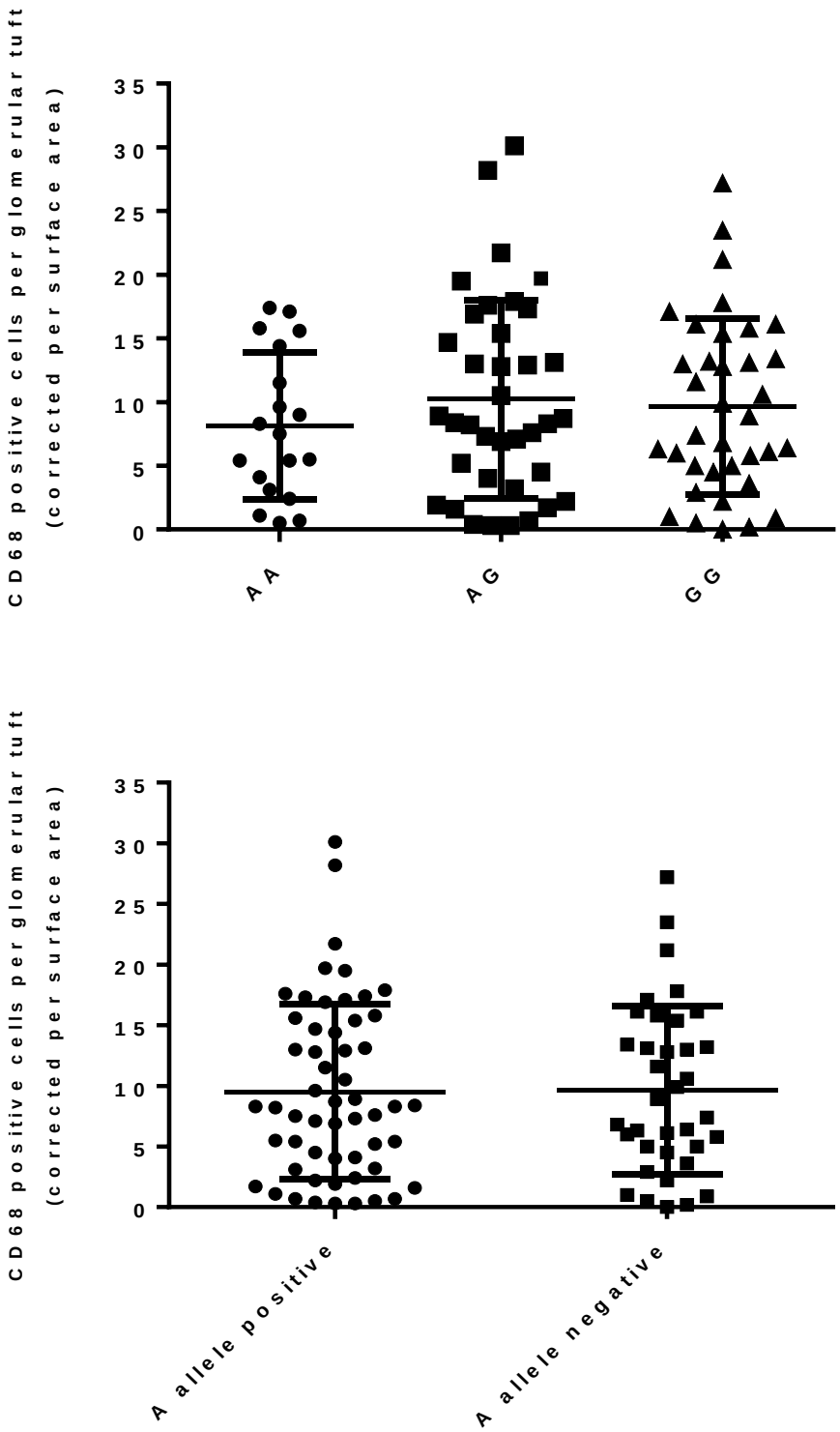
The difference in IFIH1 (rs1990760) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.27: Univariate analysis exploring clinical parameters associated with the IFIH1 (rs1990760) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	0.51	0.197 to 1.30
First degree family history	0.46	0.155 to 1.35
Juvenile onset (<18 years)	1.04	0.511 to 2.10
ESRD	0.62	0.212 to 1.80
ANA	0.22	0.0252 to 1.83
Anti-dsDNA	1.07	0.534 to 2.15
Anti-Ro	0.50	0.256 to 0.989
Anti-RNP	0.18****	0.0849 to 0.366
Anti-Sm	0.11****	0.0379 to 0.301
APS	0.89	0.388 to 2.06
Anti-cardiolipin	0.66	0.317 to 1.38
Lupus anticoagulant	0.93	0.466 to 1.87
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=92) Variable absent (n=68)	 25.1 ± 11.3 26.3 ± 10.9	0.441
Duration of follow-up (years) Variable present (n=92) Variable absent (n=68)	 12.9 ± 7.8 10.0 ± 6.4	*0.025
Glomerular CD68 count Variable present (n=56) Variable absent (n=36)	 9.5 ± 7.1 9.6 ± 6.9	0.933

****p=<0.0001, otherwise none statistically significant

Figure 4.15: CD68 immunostaining in IFIH1 (rs1990760) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative.

4.3.12 Discussion of IFIH1 genotyping results in lupus nephritis

IFIH1 (interferon induced with helicase C domain 1) also known as *MDA5* is a cytosolic sensor of double-stranded RNA that promotes type I interferon production. Heterozygous gain-of-function mutations in *IFIH1* have recently been described in which patients have an upregulated type I interferon response, neurological deficits and 'lupus-like' features (Rice et al., 2014). *IFIH1* now joins the list of genes causing so-called interferonopathies including; *TREX1, SAMDH, ADAR1, RNASEH2A, RNASEH2B* and *RNASEH2C* (Crow et al., 2006; Rice et al., 2013).

The *IFIH1* rs1990760 polymorphism was associated with SLE in a large scale replication study of European SLE patients (Cunninghame Graham et al., 2011). Variants in *IFIH1* have also been associated with other autoimmune diseases including type I diabetes and Graves' disease (Smyth et al., 2006; Sutherland et al., 2007). Robinson et al reported that the *IFIH1* rs1990760 risk allele was associated with the presence of anti-dsDNA antibodies in African and European SLE patients and that those who carried the risk allele and were anti-dsDNA antibody positive had increased sensitivity to type I interferon signalling (Robinson et al., 2011).

In our study, European lupus nephritis patients were more likely to be homozygous for the risk allele than 1,000 Genomes Project ancestral matched control individuals but the overall allelic frequency of the risk variant was not significantly higher. Patients carrying the *IFIH1* risk variant did not have a higher frequency of familial nephritis, juvenile onset disease or progression to ESRD. Anti-dsDNA antibodies were not significantly more prevalent in those with the risk allele. RNA containing autoantibodies, anti-Sm and anti-RNP, in particular, were significantly less prevalent in patients carrying the risk allele.

4.3.13 Genes involved in NFκB signalling (*IRAK1* and *TNFAIP3*)

IRAK1 (rs2269368) genotyping results

Table 4.28: Association of IRAK1 (rs2269368) polymorphism with lupus nephritis

	European controls (n=201)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	2% (n=4)	3.3% (n=2)	0.949	0.983	0.573 to 1.68
AG	29% (n=58)	24.6% (n=15)			
GG	69% (n=139)	72.1% (n=44)			
	African controls (n=131)	African lupus nephritis (n=52)			
AA	25.2% (n=33)	36.5% (n=19)	0.163	0.732	0.471 to 1.14
AG	54.2% (n=71)	44.2% (n=23)			
GG	20.6% (n=27)	19.2% (n=10)			
	East Asian controls (n=103)	East Asian lupus nephritis (n=13)			
AA	40% (n=41)	46.2% (n=6)	0.713	0.850	0.357 to 2.02
AG	50% (n=52)	46.2% (n=6)			
GG	10% (n=10)	7.7% (n=1)			

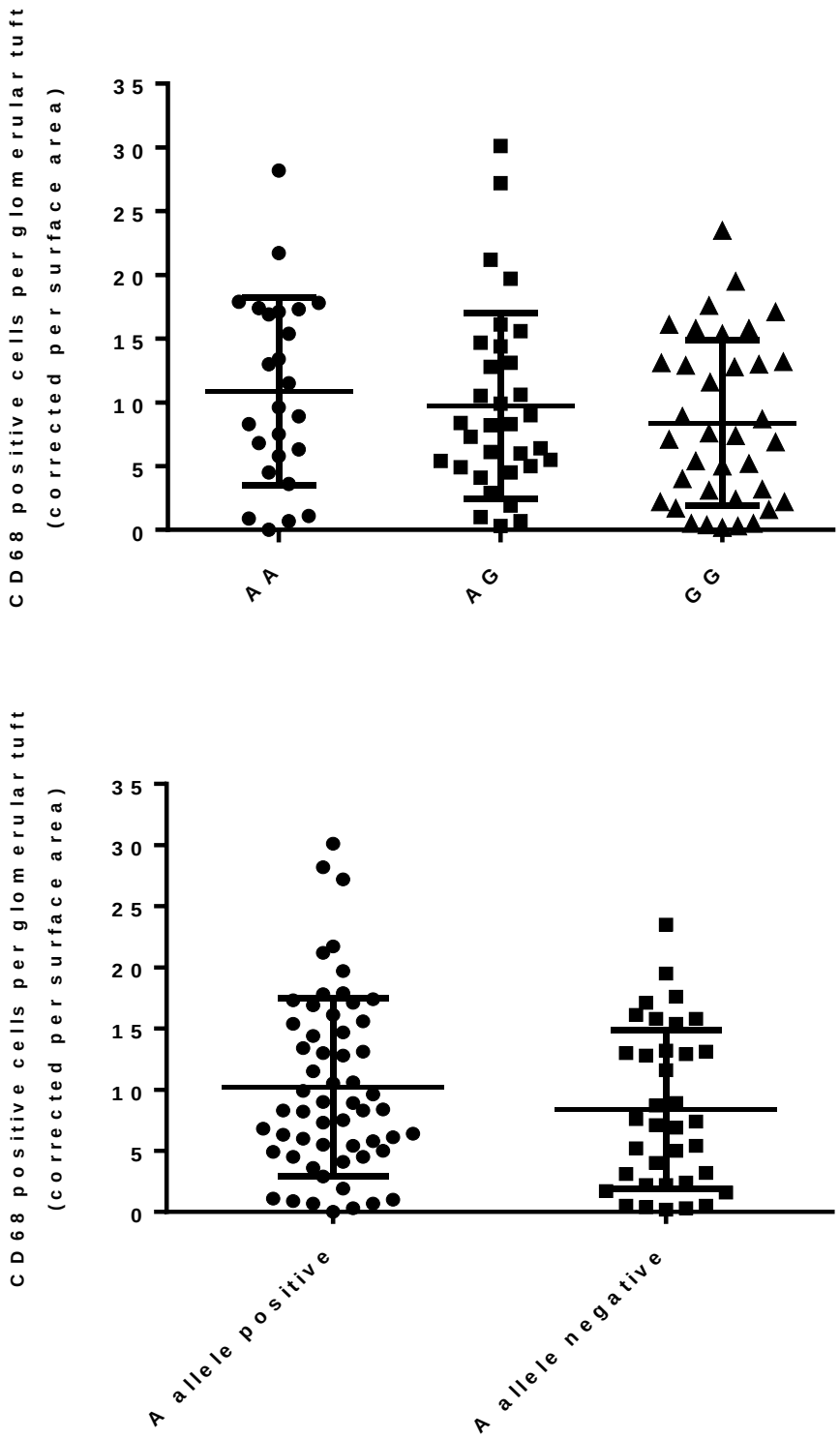
The difference in IRAK1 (rs2269368) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.29: Univariate analysis of clinical parameters associated with the IRAK1 (rs2269368) risk allele.

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	1.25	0.520 to 2.98
First degree family history	3.11	0.841 to 11.5
Juvenile onset (<18 years)	1.66	0.795 to 3.46
ESRD	2.05	0.624 to 6.76
ANA	1.08	0.233 to 4.98
Anti-dsDNA	1.11	0.554 to 2.23
Anti-Ro	1.85	0.921 to 3.70
Anti-RNP	1.88	0.939 to 3.78
Anti-Sm	1.81	0.762 to 4.31
APS	0.59	0.254 to 1.34
Anti-cardiolipin	0.91	0.433 to 1.91
Lupus anticoagulant	0.83	0.415 to 1.68
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=94) Variable absent (n=66)	 24.4 ± 10.1 27.4 ± 12.4	0.190
Duration of follow-up (years) Variable present (n=94) Variable absent (n=66)	 11.1 ± 7.2 12.6 ± 7.5	0.193
Glomerular CD68 count Variable present (n=56) Variable absent (n=36)	 10.3 ± 7.3 8.4 ± 6.5	0.197

None statistically significant

Figure 4.16: CD68 immunostaining in IRAK1 (rs2269368) polymorphism in proliferative lupus nephritis



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative.

TNFAIP3 (rs6920220) and (rs5029939) genotyping results

Table 4.30: Association of TNFAIP3 (rs6920220) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	2.4% (n=9)	11.5% (n=7)	**0.0014	0.499	0.324 to 0.769
AG	29.8% (n=113)	36.1% (n=22)			
GG	67.8% (n=257)	52.5% (n=32)			
	African Controls (n=246)	African lupus nephritis (n=52)			
AA	0.8% (n=2)	1.9% (n=1)	0.203	0.684	0.380 to 1.23
AG	22% (n=54)	28.8% (n=15)			
GG	77.2% (n=190)	69.2% (n=36)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	0%	0%	-	-	-
AG	0.5% (n=1)	0%			
GG	99.5% (n=196)	100% (n=13)			

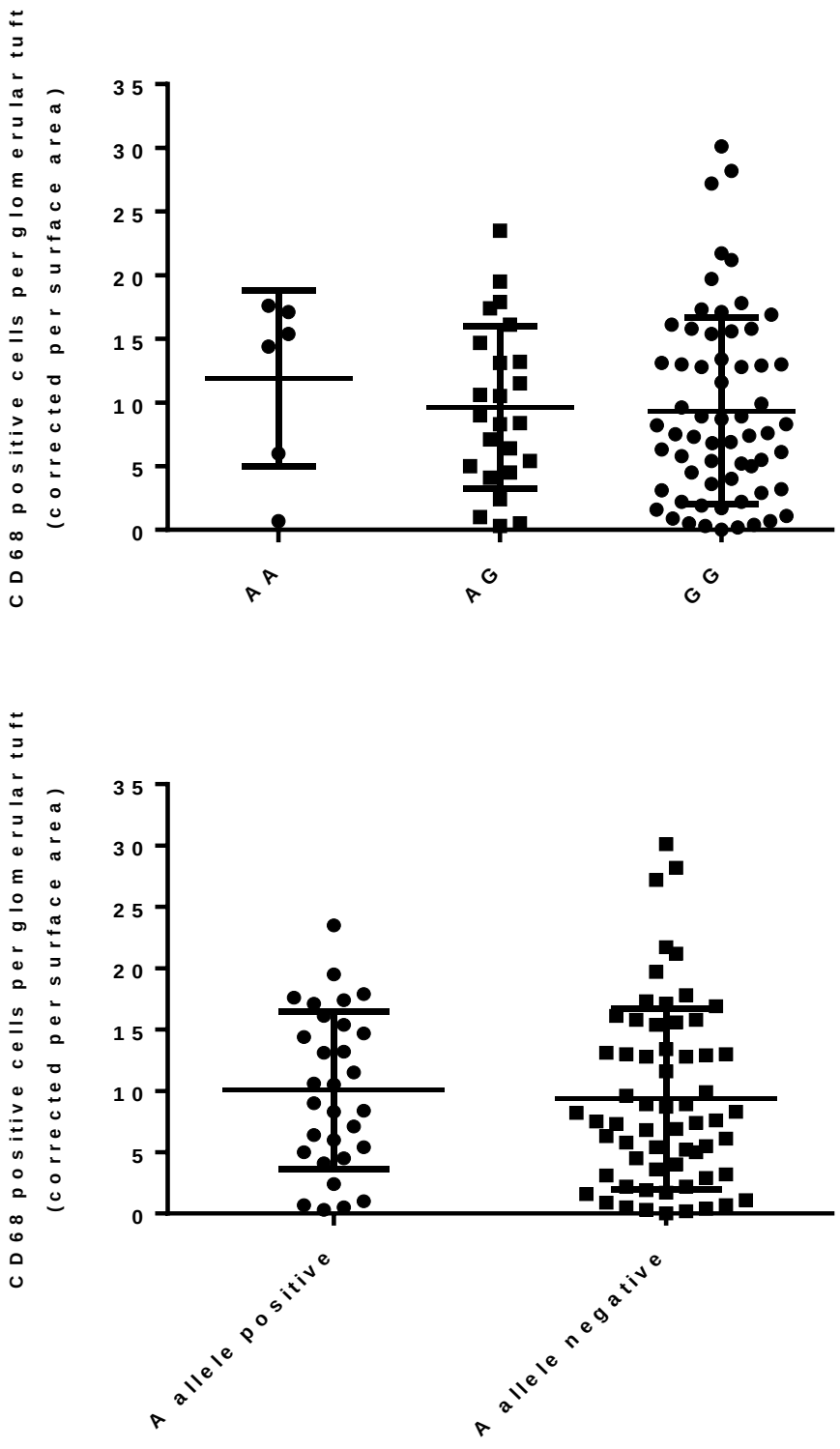
The difference in TNFAIP3 (rs6920220) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.31: Univariate analysis exploring clinical parameters associated with the TNFAIP3 (rs6920220) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	1.06	0.421 to 2.65
First degree family history	1.31	0.440 to 3.88
Juvenile onset (<18 years)	0.77	0.365 to 1.64
ESRD	1.31	0.440 to 3.88
ANA	1.31	0.246 to 7.01
Anti-dsDNA	0.66	0.326 to 1.35
Anti-Ro	1.47	0.734 to 2.95
Anti-RNP	0.67	0.326 to 1.37
Anti-Sm	0.99	0.421 to 2.32
APS	0.61	0.241 to 1.55
Anti-cardiolipin	0.74	0.333 to 1.63
Lupus anticoagulant	0.80	0.380 to 1.67
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=55) Variable absent (n=105)	 27.3 ± 11.6 24.7 ± 10.8	0.210
Duration of follow-up (years) Variable present (n=55) Variable absent (n=105)	 11.1 ± 7.5 12.1 ± 7.3	0.325
Glomerular CD68 count Variable present (n=30) Variable absent (n=62)	 10.1 ± 6.4 9.3 ± 7.3	0.415

None statistically significant

Figure 4.17: CD68 immunostaining inTNFAIP3 (rs6920220) in lupus nephritis



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative. Classes I, II and V were excluded.

Table 4.32: Association of TNFAIP3 (rs5029939) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	0%	0%	*0.014	0.332	0.132 to 0.831
AG	4% (n=15)	11.5% (n=7)			
GG	96% (n=364)	88.5% (n=54)			
	African Controls (n=246)	African lupus nephritis (n=52)			
AA	13.8% (n=34)	21.2% (n=11)	0.475	0.855	0.556 to 1.32
AG	47.6% (n=117)	40.4% (n=21)			
GG	38.6% (n=95)	38.5% (n=20)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	0%	0%	0.469	0.574	0.126 to 2.62
AG	9.1% (n=18)	15.4% (n=2)			
GG	90.9% (n=179)	84.6% (n=11)			

The difference in TNFAIP3 (rs5029939) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.33: Univariate analysis exploring clinical parameters associated with the TNFAIP3 (rs5029939) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	2.71	0.879 to 8.38
First degree family history	2.08	0.708 to 6.08
Juvenile onset (<18 years)	1.09	0.514 to 2.30
ESRD	0.78	0.236 to 2.59
ANA	1.14	0.213 to 6.10
Anti-dsDNA	0.77	0.369 to 1.60
Anti-Ro	1.51	0.746 to 3.05
Anti-RNP	3.47***	1.68 to 7.17
Anti-Sm	5.02***	2.13 to 11.9
APS	0.23*	0.0668 to 0.818
Anti-cardiolipin	0.61	0.261 to 1.40
Lupus anticoagulant	0.81	0.380 to 1.72
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=50) Variable absent (n=110)	 24.1 ± 9.6 26.3 ± 11.8	0.382
Duration of follow-up (years) Variable present (n=50) Variable absent (n=110)	 10.4 ± 7.0 12.2 ± 7.4	0.146
Glomerular CD68 count Variable present (n=26) Variable absent (n=66)	 8.9 ± 8.0 9.8 ± 6.6	0.572

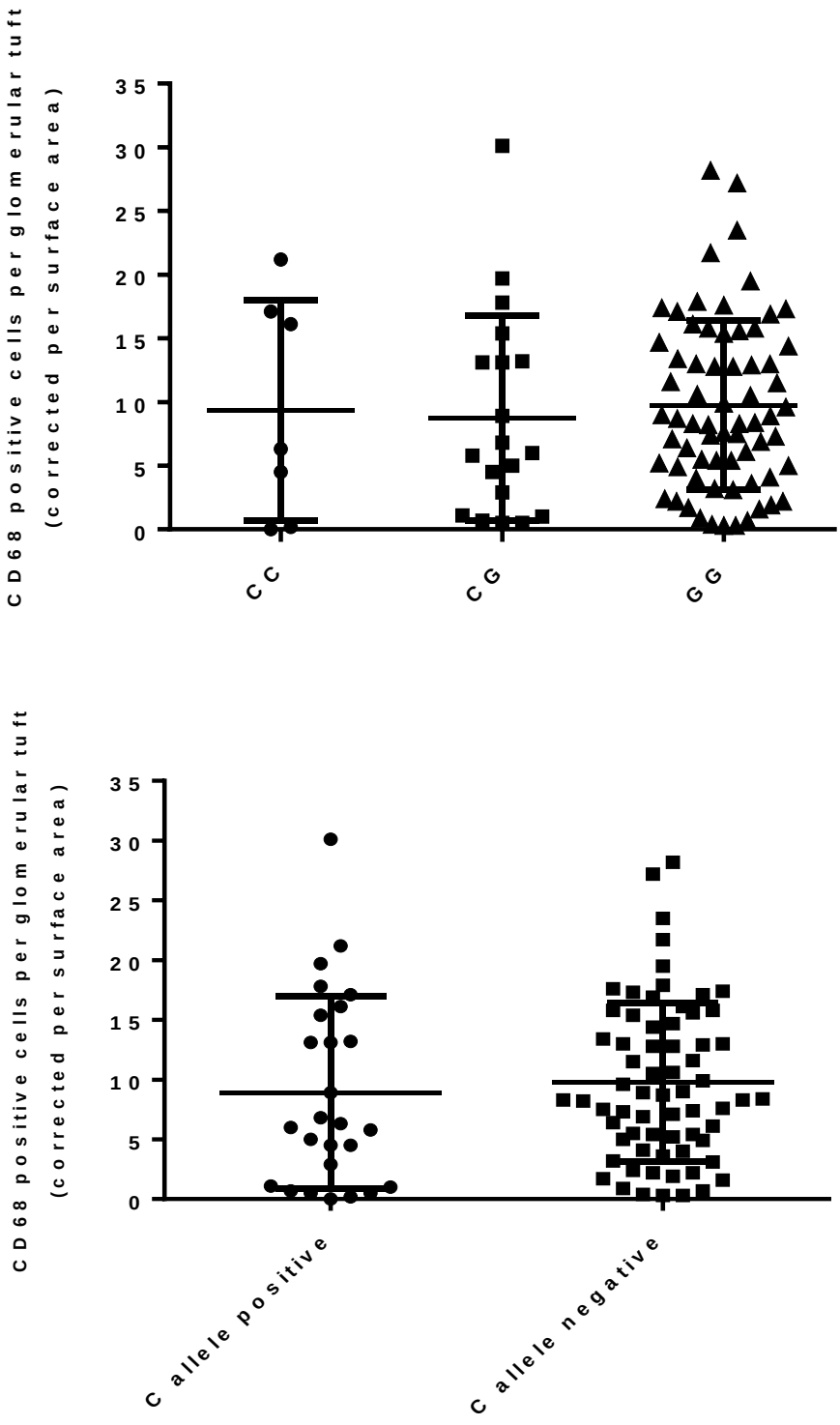
****p*=0.0006

****p*=0.0001

**p*=0.015

Otherwise none statistically significant

Figure 4.18: CD68 immunostaining in TNFAIP3 (rs5029939) in proliferative lupus nephritis



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between CC, CG and GG genotypes nor was there a difference between those who were C allele positive or negative.

4.3.14 Discussion of genes involved in NFκB signalling (IRAK1 **and** *TNFAIP3 genotyping results in lupus nephritis*

IRAK1 (interleukin-1 receptor associated kinase 1) is a serine/threonine protein kinase that is involved in TLR and IL-1 receptor signalling pathways. Irak1 deficient mice have decreased IgG and IgM autoantibodies to dsDNA and histone, reduced renal inflammation and lymphopenia (Jacob et al., 2009). Given that it is located on the X chromosome, it has been suggested that *IRAK1* polymorphisms may explain, at least in part, the predominance of female patients developing SLE.

Candidate gene studies in SLE have identified *IRAK1* as a lupus susceptibility gene in both childhood-onset and adult-onset disease in multiple ancestral groups (Jacob et al., 2007, 2009; Sanchez et al., 2011; Kaufman et al., 2013; Zhai et al., 2013).

We did not find an association of the *IRAK1* rs2269368 risk allele with lupus nephritis in our cohort. Carriers of the risk variant did seem to represent a more severe clinical phenotype, however, as they were more likely to have juvenile-onset disease and progress to ESRD (OD 1.66, 95% CI 0.795 to 3.46 and OD 2.05, 95% CI 0.624 to 6.76, respectively). Familial nephritis was seen at a higher frequency in patients with the risk allele (OD 3.11, 95% CI 0.841 to 11.5). Anti-Ro, anti-Sm and anti-RNP antibodies were more prevalent in those who carried the risk variant.

TNFAIP3 (tumour necrosis factor, alpha-induced protein 3) also known as A20, encodes an ubiquitin-editing enzyme that acts a negative regulator of NF-κB signaling and mediates TNFR, IL1R and TLR pathways (Heyninck et al., 1999; Boone et al., 2004; Wertz et al., 2004). A20 deficient mice have dysregulated TNF-induced apoptosis and multi-organ inflammation (Lee et al., 2000).

TNFAIP3 polymorphisms have been associated with SLE in European and Han Chinese GWAS (Graham et al., 2008; Han et al., 2009; Adrianto et al., 2011; Yang et al., 2012; (Martin et al., 2013). Variants in *TNFAIP3* have also been associated with other autoimmune and inflammatory disease such as rheumatoid arthritis, multiple sclerosis and type I diabetes (Plenge et al., 2007; Thomson et al., 2007; De Jager et al., 2009; Fung et al., 2009).

We examined two *TNFAIP3* polymorphisms, in our study, rs6920220 and rs5029939, both of which were found to be associated with lupus nephritis in European patients. No associations were seen with African or East Asian patients. Individuals with the *TNFAIP3* rs6920220 risk allele were more likely to progress to ESRD (OD 1.31, 95% CI 0.44 to 3.88). Anti-Ro antibody positivity was higher in those who carried the risk variant (OD 1.47, 95% CI 0.734 to 2.95).

The *TNFAIP3* rs5029939 risk allele was more frequently present in familial nephritis than sporadic disease (OD 2.08, 95% CI 0.708 to 6.08). RNA-containing autoantibodies, particularly anti-RNP and anti-Sm, were more prevalent in those carrying the risk allele (OD 3.47, 95% CI 1.68 to 7.17; p=0.0006 and OD5.02, 95% CI 2.13 to 11.9; (p=0.0001, respectively).

4.3.15 Genotyping results of genes involved in B-cell receptor signalling *(BANK1/BLK/LYN)*

BLK (rs2736340) genotyping results

Table 4.34: Association of BLK (rs2736340) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	6.9% (n=26)	13.1% (n=8)	*0.023	0.624	0.415 to 0.939
AG	35.6% (n=135)	42.6% (n=26)			
GG	57.5% (n=218)	44.3% (n=27)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	2.9% (n=7)	3.8% (n=2)	0.178	0.681	0.388 to 1.19
AG	20.7% (n=51)	28.8% (n=15)			
GG	76.4% (n=188)	67.3% (n=35)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	53.8% (n=106)	75% (n=11)	*0.037	0.239	0.0554 to 1.03
AG	40.6% (n=80)	16.7% (n=2)			
GG	5.6% (n=11)	0%			

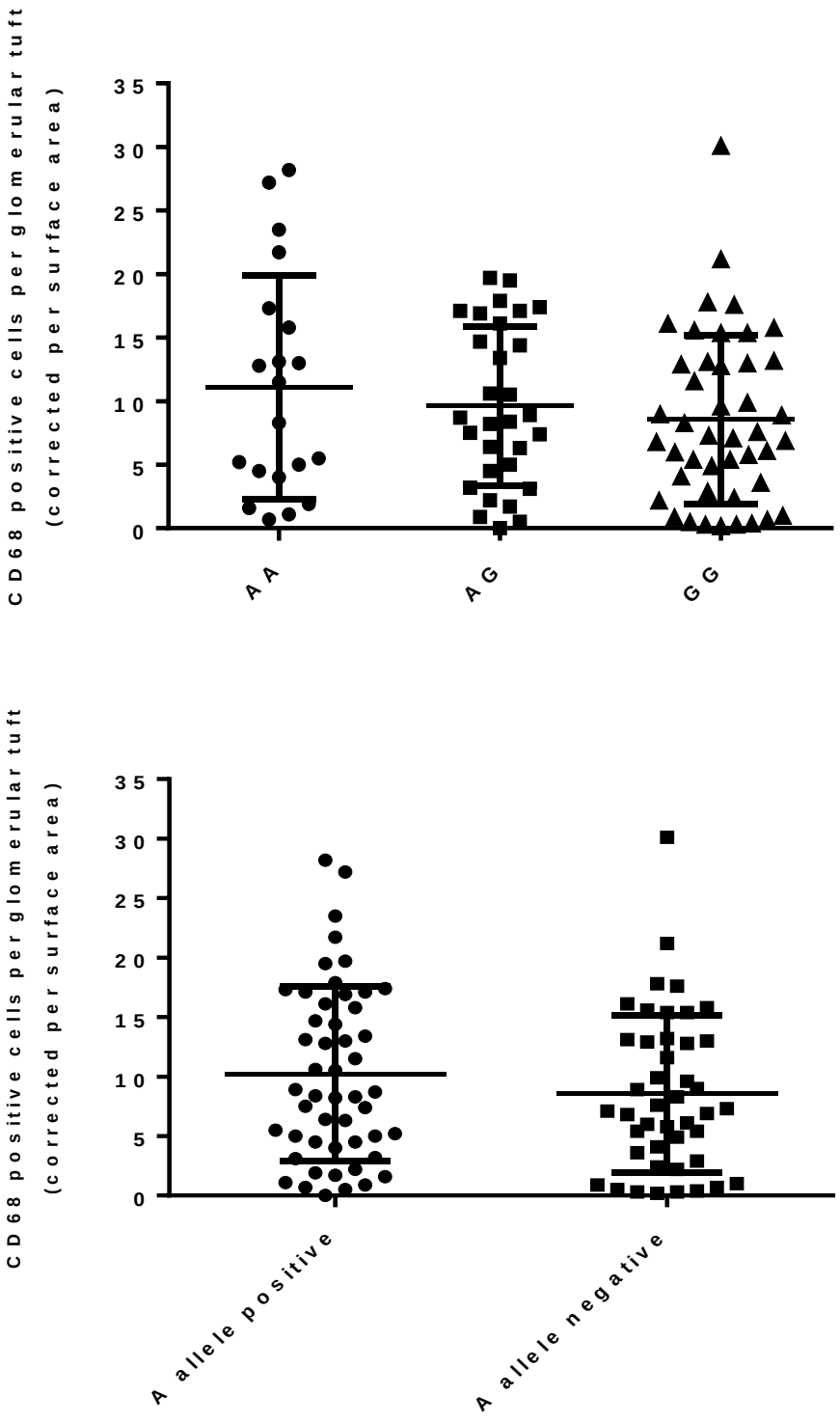
The difference in BLK (rs2736340) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.35: Univariate analysis exploring clinical parameters associated with the BLK (rs2736340) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	0.48	0.186 to 1.22
First degree family history	0.65	0.225 to 1.90
Juvenile onset (<18 years)	0.98	0.483 to 1.97
ESRD	1.19	0.401 to 3.50
ANA	0.52	0.0968 to 2.74
Anti-dsDNA	0.77	0.383 to 1.56
Anti-Ro	0.45*	0.226 to 0.881
Anti-RNP	0.65	0.331 to 1.27
Anti-Sm	0.69	0.304 to 1.55
APS	0.95	0.413 to 2.19
Anti-cardiolipin	0.94	0.450 to 1.96
Lupus anticoagulant	1.68	0.824 to 3.43
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=90) Variable absent (n=70)	 25.9 ± 12.1 25.2 ± 10.0	0.952
Duration of follow-up (years) Variable present (n=90) Variable absent (n=70)	 12.2 ± 7.6 11.1 ± 6.9	0.458
Glomerular CD68 count Variable present (n=50) Variable absent (n=42)	 10.2 ± 7.3 8.6 ± 6.7	0.295

*p=0.02, otherwise none statistically significant

Figure 4.19: CD68 immunostaining in BLK (rs2736340) in proliferative lupus nephritis



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative.

BANK1 (rs10516487) genotyping results

Table 4.36: Association of BANK1 (rs10516487) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
GG	6.9% (n=26)	8.2% (n=5)	0.585	1.13	0.732 to 1.74
AG	43.5% (n=165)	36.1% (n=22)			
AA	49.6% (n=188)	55.7% (n=34)			
	African Controls (n=246)	African lupus nephritis (n=52)			
GG	4.9% (n=12)	0%	*0.045	1.81	1.01 to 3.25
AG	37% (n=91)	28.8% (n=15)			
AA	58.1% (n=143)	71.2% (n=37)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
GG	1.5% (n=3)	0%	0.086	4.94	0.657 to 37.1
AG	29.9% (n=59)	7.7% (n=1)			
AA	68.5% (n=135)	92.3% (n=12)			

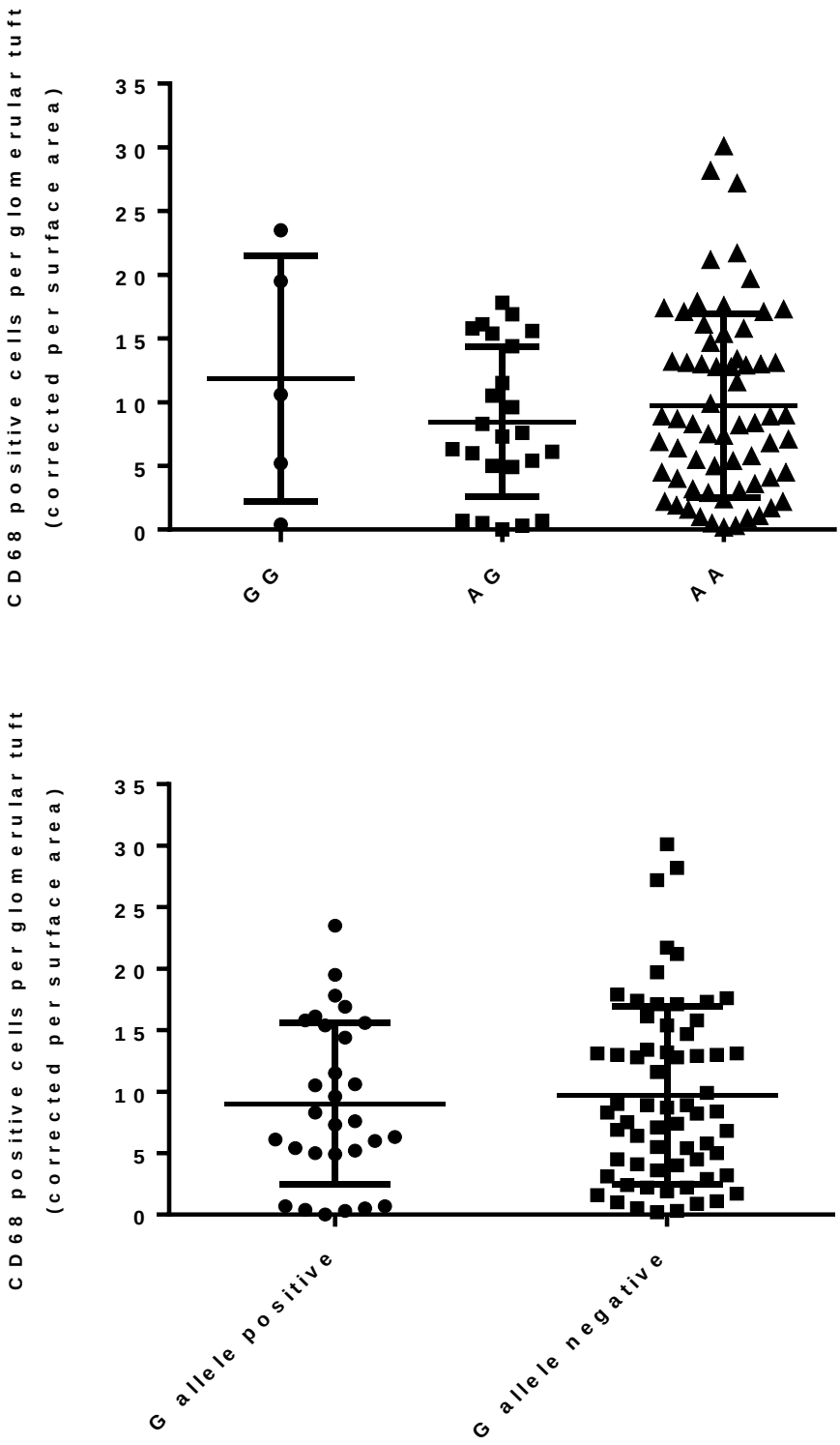
The difference in BANK1 (rs10516487) allele distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.37: Univariate analysis exploring clinical parameters associated with the BANK1 (rs10516487) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	0.72	0.291 to 1.78
First degree family history	0.06**	0.00357 to 1.04
Juvenile onset (<18 years)	0.81	0.372 to 1.74
ESRD	0.52	0.140 to 1.94
ANA	7.46	0.418 to 133
Anti-dsDNA	1.11	0.528 to 2.34
Anti-Ro	0.47	0.222 to 0.990
Anti-RNP	0.53	0.254 to 1.11
Anti-Sm	0.85	0.353 to 2.02
APS	1.11	0.460 to 2.68
Anti-cardiolipin	0.86	0.385 to 1.91
Lupus anticoagulant	1.08	0.517 to 2.26
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=50) Variable absent (n=110)	 25.7 ± 11.1 25.5 ± 11.2	0.937
Duration of follow-up (years) Variable present (n=50) Variable absent (n=110)	 11.7 ± 7.2 11.7 ± 7.4	0.909
Glomerular CD68 count Variable present (n=28) Variable absent (n=64)	 9.2 ± 6.7 9.7 ± 7.2	0.795

**p=0.0032, otherwise none statistically significant

Figure 4.20: CD68 immunostaining in BANK1 (rs10516487) in proliferative lupus nephritis



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between GG, AG and AA genotypes nor was there a difference between those who were G allele positive or negative.

LYN (rs7829816) genotyping results

Table 4.38: Association of LYN (rs7829816) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
GG	1.9% (n=7)	1.6% (n=1)	0.567	0.866	0.530 to 1.42
AG	29.8% (n=113)	34.4% (n=21)			
AA	68.3% (n=259)	63.9% (n=39)			
	African controls (n=246)	African lupus nephritis (n=52)			
GG	24% (n=59)	26.9% (n=14)	0.205	0.760	0.497 to 1.16
AG	48% (n=118)	55.8% (n=29)			
AA	28% (n=69)	17.3% (n=9)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
GG	0%	0%	-	-	-
AG	6.1% (n=12)	0%			
AA	93.9% (n=185)	100% (n=13)			

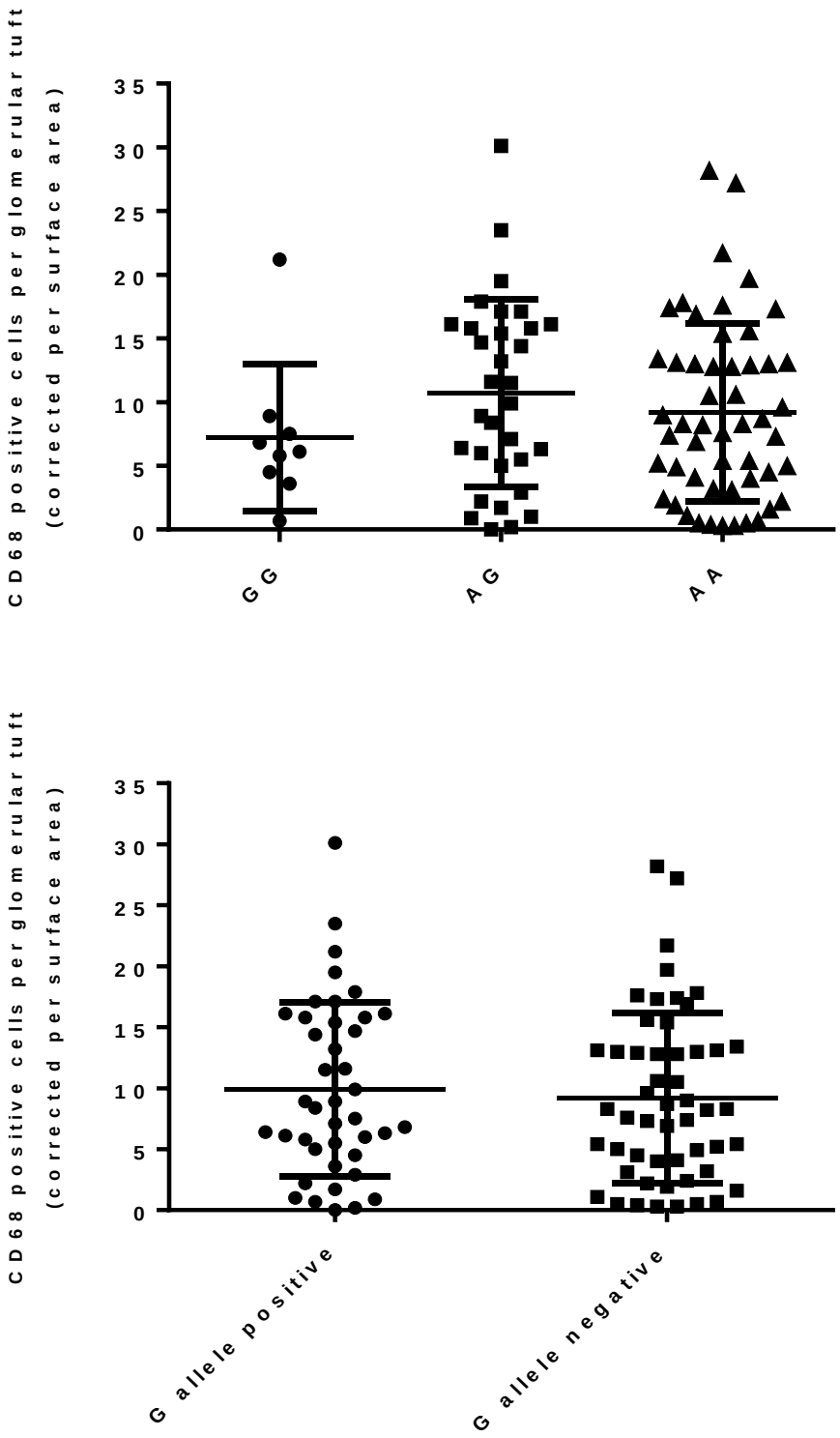
The difference in LYN (rs7829816) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.39: Univariate analysis exploring clinical parameters associated with the LYN (rs7829816) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	0.69	0.288 to 1.64
First degree family history	2.53	0.824 to 7.78
Juvenile onset (<18 years)	0.89	0.442 to 1.80
ESRD	0.76	0.255 to 2.23
ANA	2.22	0.417 to 11.8
Anti-dsDNA	0.71	0.356 to 1.42
Anti-Ro	1.12	0.575 to 2.19
Anti-RNP	2.33*	1.18 to 4.62
Anti-Sm	2.15	0.934 to 4.95
APS	1.59	0.693 to 3.67
Anti-cardiolipin	1.23	0.590 to 2.55
Lupus anticoagulant	1.41	0.706 to 2.81
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=74) Variable absent (n=86)	 26.4 ± 11.3 25.0 ± 11.0	0.429
Duration of follow-up (years) Variable present (n=74) Variable absent (n=86)	 10.7 ± 6.7 12.5 ± 7.8	0.159
Glomerular CD68 count Variable present (n=41) Variable absent (n=51)	 9.9 ± 7.1 9.3 ± 7.0	0.658

*p=0.014, otherwise none statistically significant

Figure 4.21: CD68 immunostaining in LYN (rs7829816) in proliferative lupus nephritis



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between GG, AG and AA genotypes nor was there a difference between those who were G allele positive or negative.

4.3.16 Discussion of genotyping results of genes involved in B-cell receptor signalling

BLK (B-lymphoid tyrosine kinase), *BANK1* (B-cell scaffold protein with ankyrin repeats) and *LYN* (v-yes-1 Yamaguchi sarcoma viral related oncogene homolog) are all involved in B lymphocyte activation following signalling through the B-cell receptor (BCR). BANK1 and LYN are binding partners in that the BANK1 protein mediates the interaction between LYN and the IP3 receptors leading to their phosphorylation (Yokoyama et al., 2002). Epistatic interactions have been shown between *BANK1* and *BLK* polymorphisms that increase lupus susceptibility (Castillejo-Lopez et al., 2012).

BLK polymorphisms have been shown to be associated with SLE in European and East Asian populations (Kozyrev et al, 2008; Han et al, 2009; Chung et al., 2011; Lee et al., 2012; Yang et al, 2012). *BANK1* variants have been associated in SLE GWAS in European and Chinese populations (Kozyrev et al., 2008; Chung et al., 2009). *LYN* polymorphisms have been associated with SLE with genome wide significance in European patients only. In a large replication study of African American SLE patients, *BLK* and *BANK1* polymorphisms were found to be associated with the disease (Sánchez et al, 2011). A case-control study testing 90 SNPs in *LYN* found associations with American SLE patients of European ancestry but not in patients of African American or Korean ancestry (Lu et al., 2009).

In our cohort, the *BLK* rs2736340 polymorphism was associated with lupus nephritis in patients of European and East Asian ancestry but not in patients of African ancestry. The *BANK1* rs10516487 risk allele was more commonly found in African

control data from the 1,000 Genomes Project than in our African lupus nephritis cohort. The *LYN* rs7829816 risk allele was not associated with lupus nephritis in any of the ancestral groups in our study.

Lupus nephritis patients carrying the *BANK1* rs10516487 risk allele were significantly more likely to have sporadic onset than familial disease. Individuals with the *BANK1* rs10516487 variant were more frequently ANA positive than those without the risk allele (OD 7.46, 95% CI 0.418 to 133).

Patients with the *BLK* rs2736340 risk allele were significantly less likely to be anti-Ro antibody positive than those without the risk variant. Carriers of the risk allele were marginally more likely to progress to ESRD (OD 1.19 95% CI 0.401 to 3.50) and were more often lupus anticoagulant positive (OD 1.68, 95% CI 0.824 to 3.43).

LYN rs7829816 risk allele carriers were more likely to have familial lupus nephritis (OD 2.53, 95% CI 0.824 to 7.78). ANA positivity was higher in patients with the risk allele as was the coexistence of Antiphospholipid syndrome, anti-cardiolipin antibody and lupus anticoagulant. RNA-containing autoantibodies anti-Ro, anti-RNP and anti-Sm were all more frequent in *LYN* risk allele carriers.

No associations were observed with the CD68 glomerular count and with any of the different *BANK1, BLK, LYN* genotypes or in those with or without the risk allele.

4.3.17 PTPN22 (rs2476601) genotyping results

Table 4.40: Association of PTPN22 (rs2476601) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	0.5% (n=2)	1.6% (n=1)	0.590	0.847	0.462 to 1.55
AG	18.7% (n=71)	19.7% (n=12)			
GG	80.7% (n=306)	78.7% (n=48)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	0%	0%	0.467	0.420	0.0377 to 4.68
AG	0.8% (n=2)	1.9% (n=1)			
GG	99.2% (n=244)	98.1% (n=51)			
	East Asian Controls (n=197)	East Asian lupus nephritis (n=13)			
AA	0%	0%	-	-	-
AG	0%	0%			
GG	100% (n=197)	100% (n=13)			

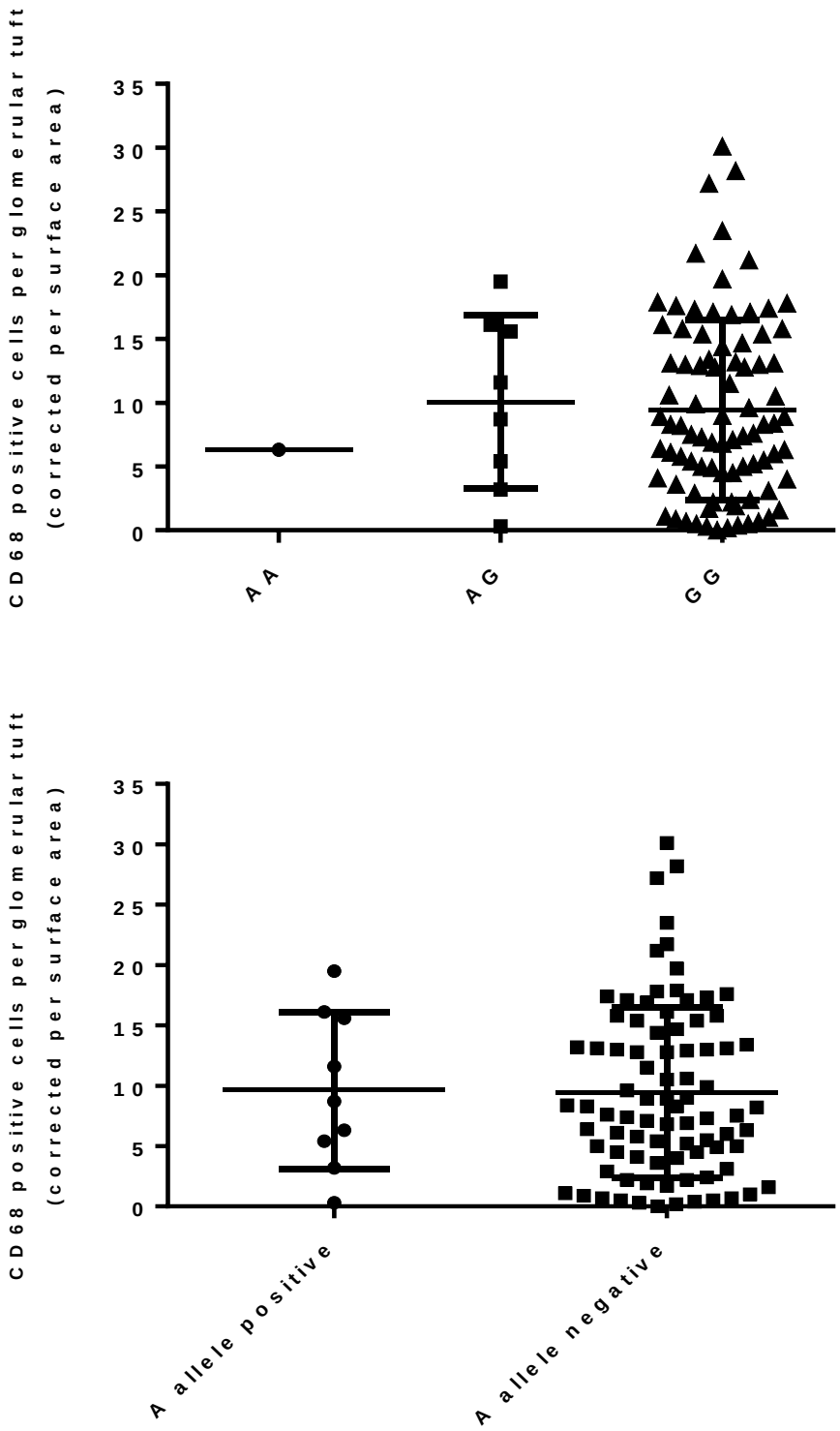
The difference in PTPN22 (rs2476601) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.41: Univariate analysis exploring clinical parameters associated with the PTPN22 (rs2476601) risk allele

Categorical Variables	**Odds ratio**	**95% Confidence Interval**
Gender (female)	1.26	0.268 to 5.95
First degree family history	0.25	0.0145 to 4.43
Juvenile onset (<18 years)	2.33	0.810 to 6.72
ESRD	0.57	0.0706 to 4.65
ANA	0.66	0.0745 to 5.88
Anti-dsDNA	0.50	0.173 to 1.43
Anti-Ro	0.75	0.245 to 2.27
Anti-RNP	0.52	0.160 to 1.71
Anti-Sm	0.55	0.118 to 2.57
APS	1.74	0.515 to 5.87
Anti-cardiolipin	0.20	0.0255 to 1.58
Lupus anticoagulant	1.68	0.562 to 5.04
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=16) Variable absent (n=144)	 21.2 ± 6.5 26.1 ± 11.5	0.114
Duration of follow-up (years) Variable present (n=16) Variable absent (n=144)	 14.1 ± 6.6 11.4 ± 7.4	0.092
Glomerular CD68 count Variable present (n=8) Variable absent (n=84)	 10.1 ± 6.8 9.5 ± 7.1	0.762

None statistically significant

Figure 4.22: CD68 immunostaining in PTPN22 (rs2476601) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were risk allele positive or negative.

4.3.18 Discussion of PTPN22 genotyping results in lupus nephritis

PTPN22 encodes the protein Lyp, a tyrosine phosphatase. Its primary function is in the regulation of T-cell activation by inhibiting Src and Syk family kinase signalling in conjunction with Csk (Stanford et al., 2012). The lupus associated *PTPN22* 1858C>T polymorphism affects the bindings of Lyp to Csk, resulting in abnormal regulation of T-cell activation (Fiorillo et al., 2010; Vang et al., 2012).

The *PTPN22* 1858C>T polymorphism is one of the most strongly associated genetic risk factors for autoimmunity outside of the HLA region. In addition to SLE, this variant is also associated with several other autoimmune diseases including rheumatoid arthritis, type I diabetes mellitus, Hashimoto's thyroiditis, Graves' disease and juvenile inflammatory arthritis (Lee et al., 2007). The frequency of the 1858T risk allele varies significantly between different ancestral groups. It is most commonly seen in those of Northern European origin and is rarely present in African and Asian populations. In keeping with this, our study showed no African or East Asian lupus nephritis patients who were homozygous for the risk allele and only one African patient who was heterozygous.

The first association of the 1858T variant with SLE was in a case-control study of American patients of European ancestry (Kyogoku et al., 2004). The authors did not find any differences in clinical manifestations such as nephritis, central nervous system disease, arthritis or autoantibody prolife in SLE patients who were carrying the risk allele compared to those who were not. The 1858T variant was later found to have genome wide significance of association with SLE in a cohort of European ancestry (Gateva et al., 2009). A further GWAS of anti-dsDNA positive SLE patients confirmed the association (Chung et al., 2011).

Previously, those who have shown associations between *PTPN22* risk variants and SLE have not demonstrated a stronger association with the development of nephritis than for SLE in general (Orozco et al., 2005; Reddy et al., 2005). We did not find an association of the *PTPN22* risk allele with lupus nephritis patients in our study either.

Carriers of this risk allele in our cohort did not have a higher prevalence of autoantibodies or a more frequent progression to ESRD. They were more likely to have sporadic than familial lupus nephritis, however, and were significantly younger at disease onset than those without the risk variant.

An association has been previously reported between *PTPN22* risk variants and Antiphospholipid syndrome, anti-cardiolipin antibody and lupus anticoagulant (Ostenak et al., 2014). This was verified in a second study which showed a positive association between the 1858T variant and SLE patients who had moderate to high titres of IgG anti-cardiolipin antibody (Namjou et al., 2013). In our study, the carriers of the risk allele were also more likely to have Antiphospholipid syndrome (OD 1.74, 95% CI 0.515 to 5.87) and lupus anticoagulant (OD 1.68, 95% CI 0.562 to 5.04). No association was seen with anti-cardiolipin antibodies although antibody titres were not documented in our study.

4.3.19 TNFSF4 (rs2205960) genotyping results

Table 4.42: Association of TNFSF4 (rs2205960) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	5.2% (n=20)	9.8% (n=6)	**0.0043	0.548	0.362 to 0.832
AG	31.7% (n=120)	45.9% (n=28)			
GG	63.1% (n=239)	44.3% (n=27)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	0%	0%	0.844	0.895	0.295 to 2.72
AG	6.9% (n=17)	7.7% (n=4)			
GG	93.1% (n=229)	92.3% (n=48)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	6.6% (n=13)	7.7% (n=1)	0.360	0.677	0.293 to 1.57
AG	39.6% (n=78)	53.8% (n=7)			
GG	53.8% (n=106)	38.5% (n=5)			

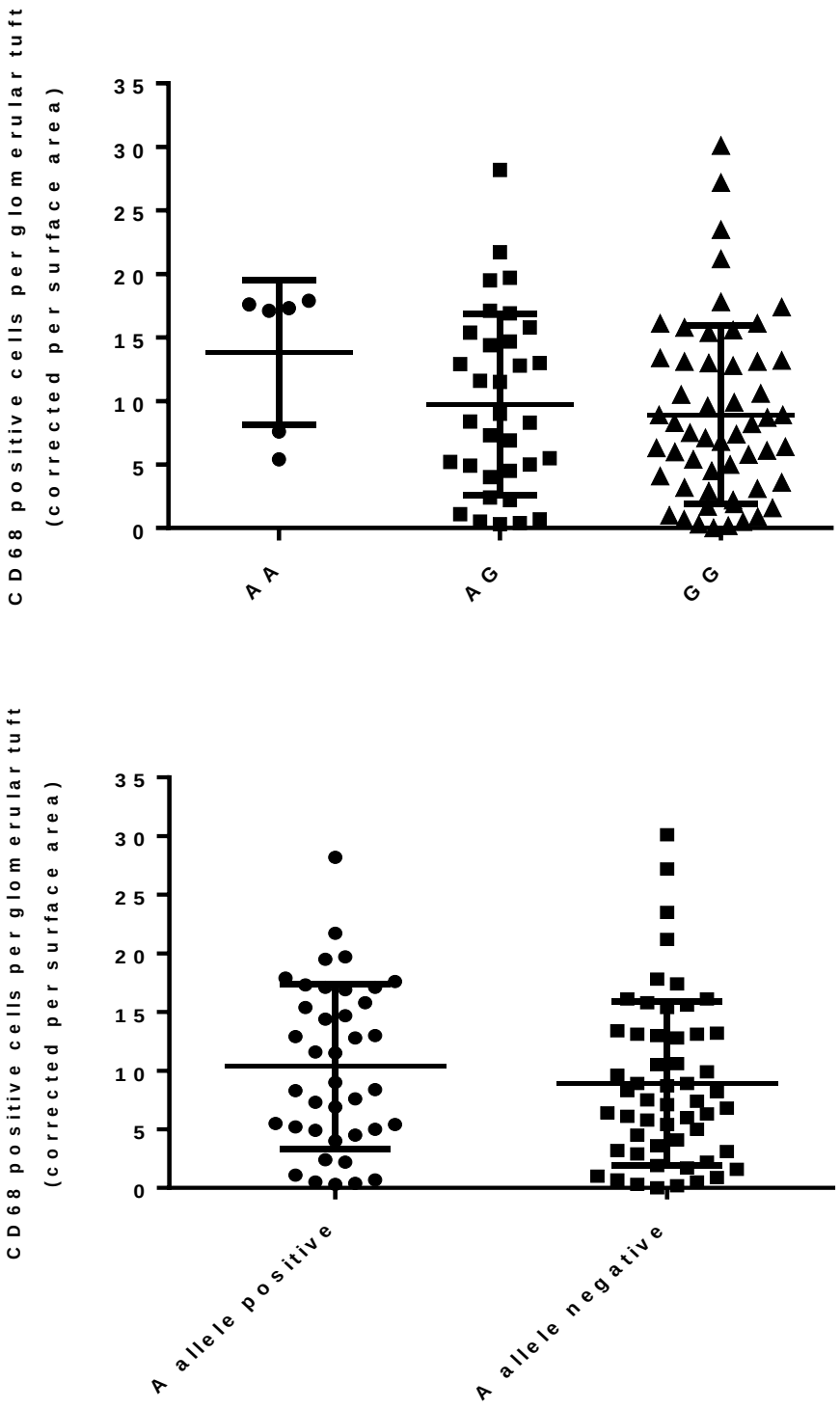
The difference in TNFSF4 (rs2205960) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project

Table 4.43: Univariate analysis exploring clinical parameters associated with the TNFSF4 (rs2205960) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	1.17	0.477 to 2.85
First degree family history	0.71	0.230 to 2.18
Juvenile onset (<18 years)	1.22	0.602 to 2.48
ESRD	0.71	0.230 to 2.18
ANA	0.51	0.110 to 2.35
Anti-dsDNA	0.76	0.381 to 1.53
Anti-Ro	0.64	0.321 to 1.28
Anti-RNP	0.55	0.276 to 1.11
Anti-Sm	0.38*	0.150 to 0.951
APS	0.99	0.428 to 2.31
Anti-cardiolipin	0.66	0.308 to 1.41
Lupus anticoagulant	1.11	0.554 to 2.23
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=65) Variable absent (n=95)	 24.0 ± 10.1 26.7 ± 11.7	0.188
Duration of follow-up (years) Variable present (n=65) Variable absent (n=95)	 12.4 ± 7.4 11.3 ± 7.3	0.304
Glomerular CD68 count Variable present (n=38) Variable absent (n=54)	 10.5 ± 7.1 8.9 ± 7.0	0.236

*p=0.03, otherwise none statistically significant

Figure 4.23: CD68 immunostaining in TNFSF4 (rs2205960) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative.

4.3.20 Discussion of TNFSF4 genotyping results in lupus nephritis

TNFSF4 (TNF superfamily, member 4) also known as OX40 ligand (OX40L) is a co-stimulatory molecule present on the surface of antigen presenting cells. Binding of OX40L to its receptor OX40 leads to activation of CD4+T lymphocytes and inhibition of regulatory T-cells (Gramaglia et al., 2000; Ito et al., 2006). OX40/OX40L signalling is also involved in activation and differentiation of B lymphocytes (Stuber et al., 1995).

Polymorphisms in *TNFSF4* were initially found to be associated with SLE in European and East Asian GWAS (Cunninghame Graham et al., 2008; Han et al., 2009; Yang et al., 2010). Subsequently associations were found in African American SLE patients (Sánchez et al, 2011; Manku et al., 2013).

The *TNFSF4* rs2205960 risk variant was strongly associated with lupus nephritis in European patients in our cohort. No association was seen with African or East Asian patients. Very few African patients in our study carried the risk allele.

It has been previously published that the *TNFSF4* rs2205960 risk allele is associated with the presence of the anti-Sm autoantibody in African and European SLE patients (Manku et al., 2013). We did not see this association in our study. In fact, we found that the anti-Sm antibody was significantly more frequent in lupus nephritis patients without the risk allele. Apart from this observation, no other significant trends were seen in autoantibody profile, disease onset, familial nephritis, prevalence of ESRD or glomerular CD68 count in those who carried the *TNFSF4* rs2205960 risk allele.

4.3.21 NCF2 (rs10911363) genotyping results

Table 4.44: Association of NCF2 (rs10911363) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	7.9% (n=30)	13.1% (n=8)	0.180	0.757	0.503 to 1.14
AC	39.6% (n=150)	40.9% (n=25)			
CC	52.5% (n=199)	45.9% (n=28)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	6.9% (n=17)	11.5% (n=6)	0.484	0.853	0.545 to 1.33
AC	48.4% (n=119)	46.2% (n=24)			
CC	44.7% (n=110)	42.3% (n=22)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	29.9% (n=59)	53.8% (n=7)	0.537	0.774	0.343 to 1.75
AC	50.8% (n=100)	15.4% (n=2)			
CC	19.3% (n=38)	30.8% (n=4)			

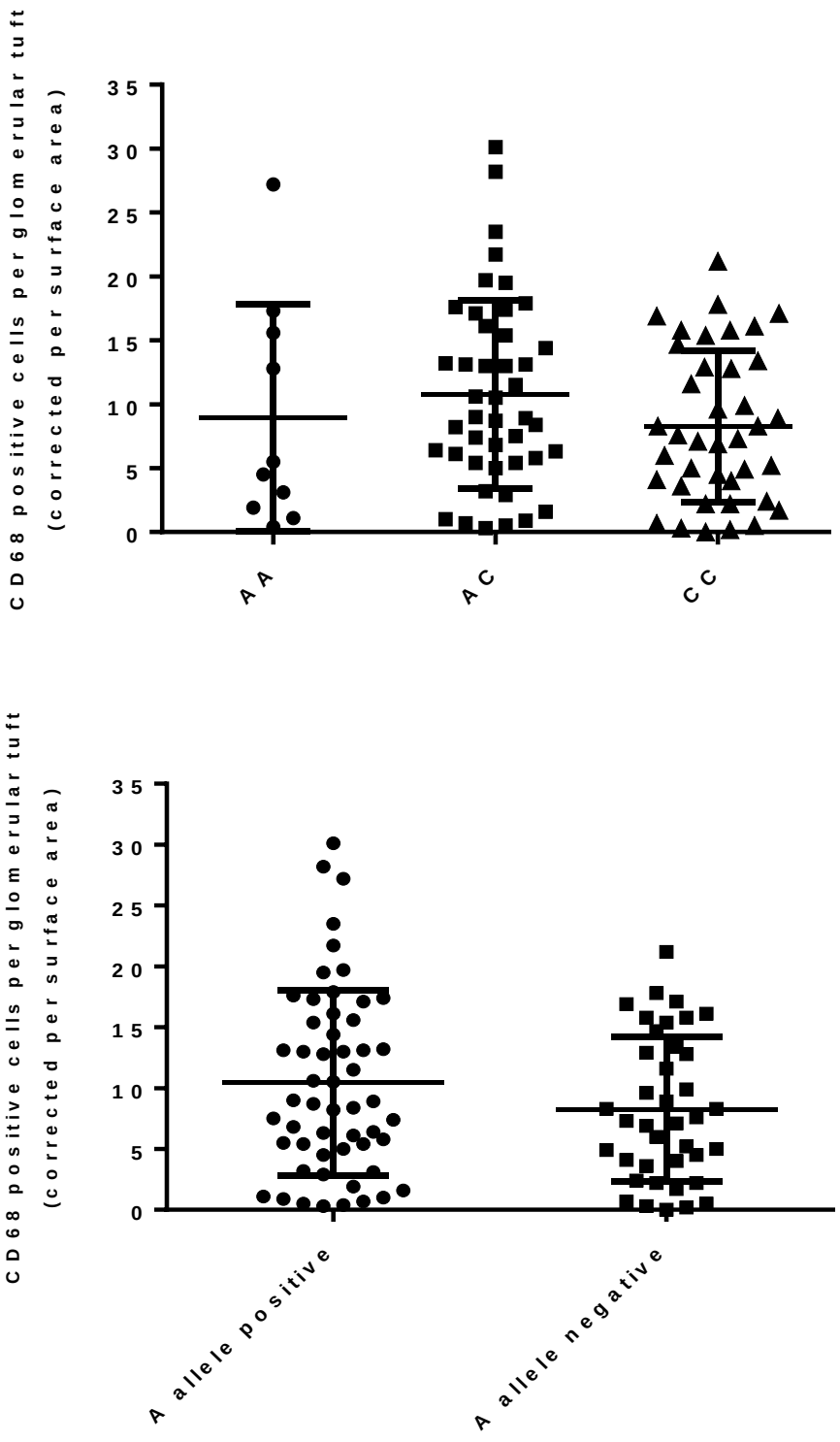
The difference in NCF2 (rs10911363) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.45: Univariate analysis exploring clinical parameters associated with the NCF2 (rs10911363) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	0.81	0.330 to 1.97
First degree family history	0.81	0.278 to 2.35
Juvenile onset (<18 years)	0.88	0.434 to 1.78
ESRD	1.09	0.368 to 3.22
ANA	1.08	0.233 to 4.98
Anti-dsDNA	1.15	0.575 to 2.32
Anti-Ro	0.63	0.319 to 1.23
Anti-RNP	0.48	0.242 to 0.945
Anti-Sm	1.01	0.441 to 2.30
APS	1.25	0.533 to 2.94
Anti-cardiolipin	1.52	0.707 to 3.25
Lupus anticoagulant	1.51	0.738 to 3.08
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=93) Variable absent (n=67)	 25.8 ± 10.8 25.3 ± 11.7	0.529
Duration of follow-up (years) Variable present (n=93) Variable absent (n=67)	 12.0 ± 7.8 11.3 ± 6.6	0.803
Glomerular CD68 count Variable present (n=54) Variable absent (n=38)	 10.4 ± 7.6 8.4 ± 6.0	0.171

None statistically significant

Figure 4.24: CD68 immunostaining in NCF2 (rs10911363) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). CD68 positivity was higher in AC genotype than CC (*p=0.0372) otherwise there was no significant difference between genotypes. There was no difference between those who were A allele positive or negative.

4.3.22 Discussion of NCF2 genotyping results in lupus nephritis

NCF2 (neutrophil cytosolic factor 2) is a component of the NADPH oxidase complex and encodes the p67 protein. Mutations in *NCF2* have previously been documented in Chronic Granulomatous Disease (CGD) (Noack et al., 1999; Patino et al., 1999). Neutrophils from *NCF2* mutated CGD patients have confirmed that the p67 protein functions in the respiratory burst mediated by NADPH oxidase (Okamura et al., 1990). *NCF2* may also play a role in production of free radicals during B-cell activation leading to increased autoantibody production (Vene et al., 2010). Interestingly, an overlap has been reported between X-linked CGD cases and lupus with co-existent diagnoses of both diseases in several case reports. Female relatives of CGD patients have an increased rate of SLE and discoid lupus (Cale et al., 2007). Furthermore, neutrophils are receiving increased recognition for their role in lupus pathogenesis given that neutrophil extracellular traps (NETs) have been identified as major producers of type I interferon through pDC activation via TLR9 (Garcia-Romo et al., 2011; Lande et al., 2011; Villaneuva et al., 2011).

A large replication study in European patients showed an association between *NCF1* rs10911363 polymorphism and SLE. We did not find any association with this polymorphism in any of the ancestral groups in our study. Carriers of the *NCF2* risk allele in our cohort were not any younger at disease onset neither did they have an increased frequency of ESRD. Antiphospholipid syndrome was more prevalent in those with the risk allele (OD 1.25, 95% CI 0.533 to 2.94). In addition, anti-cardiolipin antibody and lupus anticoagulant were more common in patients with the risk variant (OD 1.52, 95% CI 0.707 to 3.25 and OD 1.51, 95% CI 0.738 to 3.08, respectively).

4.3.23 ETS1 (rs6590330) genotyping results

Table 4.46: Association of ETS1 (rs6590330) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
AA	1.1% (n=4)	3.3% (n=2)	0.221	0.710	0.410 to 1.23
AG	19.8% (n=75)	22.9% (n=14)			
GG	79.1% (n=300)	73.8% (n=45)			
	African controls (n=246)	African lupus nephritis (n=52)			
AA	4.1% (n=10)	5.8% (n=3)	**0.0032	0.503	0.317 to 0.799
AG	32.5% (n=80)	55.8% (n=29)			
GG	63.4% (n=156)	38.5% (n=20)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
AA	13.7% (n=27)	46.2% (n=6)	*0.014	0.372	0.164 to 0.841
AG	47.2% (n=93)	30.8% (n=4)			
GG	39.1% (n=77)	23.1% (n=3)			

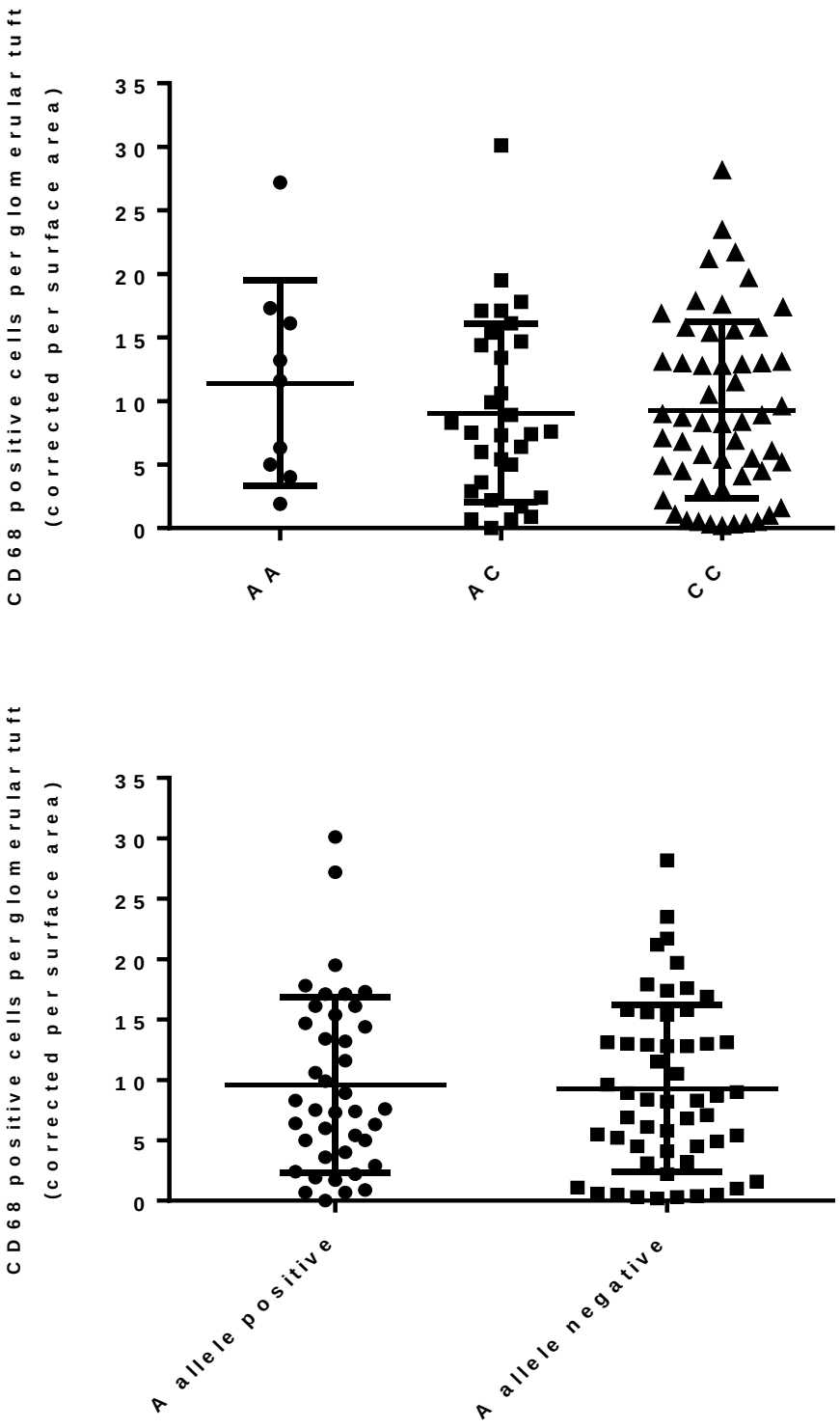
The difference in ETS1 (rs6590330) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project

Table 4.47: Univariate analysis exploring clinical parameters associated with the ETS1 (rs6590330) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	1.01	0.419 to 2.44
First degree family history	2.25	0.760 to 6.66
Juvenile onset (<18 years)	0.88	0.430 to 1.79
ESRD	0.67	0.218 to 2.06
ANA	1.89	0.355 to 10.1
Anti-dsDNA	1.02	0.508 to 2.05
Anti-Ro	1.42	0.723 to 2.79
Anti-RNP	3.02**	1.51 to 6.05
Anti-Sm	2.85*	1.23 to 6.60
APS	0.77	0.330 to 1.82
Anti-cardiolipin	1.02	0.488 to 2.15
Lupus anticoagulant	0.44*	0.209 to 0.924
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=67) Variable absent (n=93)	 27.3 ± 12.4 24.4 ± 10.4	0.286
Duration of follow-up (years) Variable present (n=67) Variable absent (n=93)	 10.1 ± 6.1 12.9 ± 8.0	0.051
Glomerular CD68 count Variable present (n=40) Variable absent (n=52)	 9.6 ± 7.3 9.6 ± 6.9	0.992

**p=0.0015, *p=0.012, *p=0.028, otherwise none statistically significant

Figure 4.25: CD68 immunostaining in ETS1 (rs6590330) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative.

4.3.24 Discussion of ETS1 genotyping results in lupus nephritis

ETS1 (V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog 1) is a member of the ETS family of transcription factors. The ETS family is defined by a conserved DNA-binding domain that recognizes the core consensus DNA sequence GGAA/T of target genes (Dwyer et al., 2007). Ets-1 plays an inhibitory role in Th17 and B-cell differentiation (Bories et al., 1995; Moisan et al., 2007). Ets-1 deficient mice develop autoimmunity with autoantibody production (anti-dsDNA, anti-histone, anti-cardiolipin), immune complex deposition and inflammatory infiltrates in the kidney (Wang et al., 2005).

Polymorphisms in *ETS1* have been shown to be associated with SLE in East Asian GWAS but have not been confirmed with genome wide significance in other ancestral groups (Han et al, 2009; Yang et al., 2010). *ELF1*, another member of the ETS family, has also been associated with East Asian SLE but was not examined in our study (Yang et al., 2011). In our cohort, there was an association between the *ETS1* rs6590330 risk allele and lupus nephritis in East Asian and African patients. The association with African lupus nephritis has not been previously reported to our knowledge.

Patients with familial lupus nephritis were more likely to carry the *ETS1* risk allele, (OD 2.25, 95% CI 0.76 to 6.66), although carriers did not have younger onset disease or increased progression to ESRD. ANA and anti-Ro positivity were more common in carriers of the risk allele. Anti-RNP and anti-Sm were significantly associated with the *ETS1* risk variant (OD 3.02, 95% CI 1.51 to 6.05 and OD 2.85, 95% CI 1.23 to 6.60, respectively).

4.3.25 RasGRP3 (rs13385731) genotyping results

Table 4.48: Association of RasGRP3 (rs13385731) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=61)	Allelic p-value	OD	95% CI
GG	0.5% (n=2)	0%	0.361	1.55	0.603 to 3.97
AG	11.3% (n=43)	8.2% (n=5)			
AA	88.1% (n=334)	91.8% (n=56)			
	African controls (n=246)	African lupus nephritis (n=52)			
GG	0.4% (n=1)	0%	0.731	0.839	0.307 to 2.29
AG	7.3% (n=18)	19.6% (n=5)			
AA	92.3% (n=227)	90.4% (n=47)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
GG	4.6% (n=9)	0%	0.451	1.60	0.467 to 5.48
AG	25.4% (n=50)	23.1% (n=3)			
AA	70% (n=138)	76.9% (n=10)			

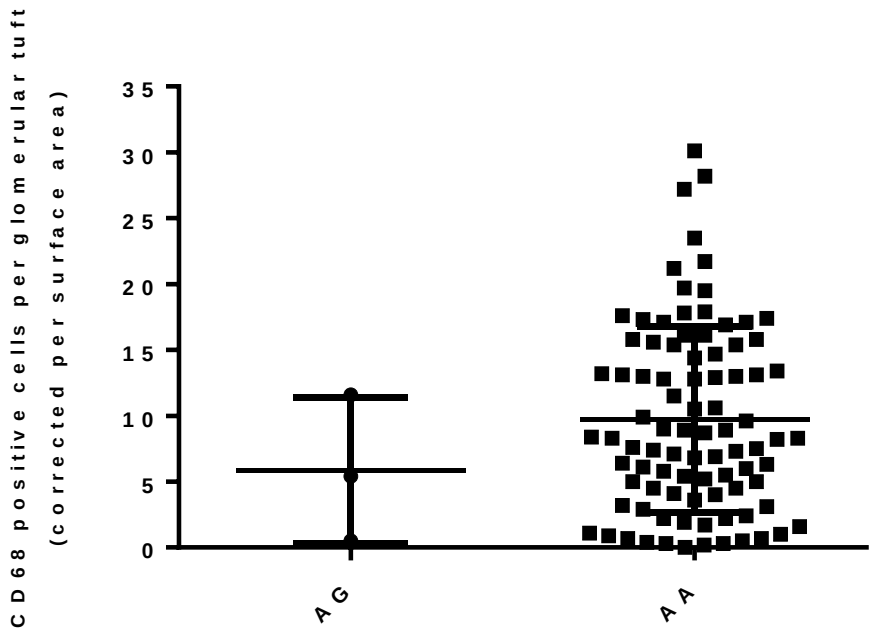
The difference in RasGRP3 (rs13385731) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project

Table 4.49: Univariate analysis exploring clinical parameters associated with the RasGRP3 (rs13385731) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	2.85	0.359 to 22.7
First degree family history	0.62	0.0759 to 5.05
Juvenile onset (<18 years)	1.27	0.414 to 3.89
ESRD	1.44	0.294 to 7.04
ANA	0.66	0.0745 to 5.88
Anti-dsDNA	1.29	0.392 to 4.22
Anti-Ro	1.79	0.593 to 5.41
Anti-RNP	1.29	0.421 to 3.92
Anti-Sm	0.65	0.138 to 3.10
APS	1.74	0.515 to 5.87
Anti-cardiolipin	0.41	0.0894 to 1.91
Lupus anticoagulant	1.50	0.511 to 4.40
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=16) Variable absent (n=144)	 25.1 ± 11.9 25.6 ± 11.1	0.748
Duration of follow-up (years) Variable present (n=16) Variable absent (n=144)	 13.7 ± 10.1 11.5 ± 7.0	0.560
Glomerular CD68 count Variable present (n=3) Variable absent (n=89)	 5.8 ± 5.6 9.7 ± 7.1	0.347

None statistically significant

Figure 4.26: CD68 immunostaining in RasGRP3 (rs13385731) polymorphism with in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There were no patients with the GG genotype within the cohort. CD68 positivity was significantly lower (**p=0.0097) in the AG genotype than those with and the AA genotype.

Table 4.50: Lupus nephritis patients carrying the RasGRP3 (rs13385731) risk allele

Gender	Ancestry	Juvenile onset	Familial	Class of nephritis
Female	African	-	-	Class V
Female	South Asian	-	-	Class II
Female	East Asian	-	-	Class V
Female	Other Ancestry	-	-	Tissue not available
Female	European	-	-	Tissue not available
Female	South Asian	-	-	Class III
Female	African	-	-	Class V
Female	European	-	-	Tissue not available
Female	African	-	-	Tissue not available
Female	Other Ancestry	-	-	Class IV-G
Female	African	-	-	Class V
Female	European	-	-	Tissue not available
Female	European	+	-	Class III
Female	European	+	-	Class V
Female	East Asian	+	-	Class V
Male	East Asian	+	+	Tissue not available
Female	African	+	-	Tissue not available

4.3.26 Discussion of RasGRP3 genotyping results in lupus nephritis

RasGRP3 (Ras guanyl nucleotide releasing protein 3) belongs to a family of guanine nucleotide-exchange factors (GEFs) that provide a link between cell surface receptors and Ras, resulting in activation of Ras and related GTPases such as Rap1 (Rebhun et al., 2000). There is increasing evidence in the literature supporting the role of the RasGRP family in the development of autoimmunity. *RasGRP1* and *RasGRP3* work in tandem, downstream of the TCR and BCR, regulating the adaptive immune response. A role for *RasGRP3* in innate immunity has also been proposed involving Ras and Rap1 stimulation during phagocytosis by macrophages (Botelho et al., 2009). Aged Rasgrp1-/- mice develop autoimmunity with ANA production and splenomegaly while Rasgrp3-/- mice exhibit hypogammaglobulinemia and decreased BCR-regulated Ras-Erk signalling (Layer et al., 2003; Coughlin et al., 2005). A number of reports of defective *RasGRP1* expression in SLE patients have also emerged (Yasuda et al., 2007; Rapoport et al., 2011).

The *RasGRP3* rs13385731 polymorphism has been associated with SLE in East Asian GWAS but has not been confirmed in other ethnicities (Han et al, 2009; He et al., 2010). No patients in our study were homozygous for the risk allele and only 13 were heterozygous. No association between the *RasGRP3* rs13385731 polymorphism and lupus nephritis was observed in our study. There were, however, a number of mutations in Ras related genes identified in our cohort by WES. These include *RASGRF2*, *RASSF5* and *RIN*. They will be discussed in more detail in a later section of this thesis.

Our lupus nephritis patients with the *RasGRP3* risk allele were more likely to progress to ESRD (OD 1.44, 95% CI 0.294 to 7.04). Anti-dsDNA, anti-Ro, anti-RNP were all more frequently seen in these patients also. Malar rash, discoid rash, serositis and ANA positivity have been associated with this variant in a previous case-only study (He et al., 2010). In our study group Antiphospholipid syndrome and lupus anticoagulant were more prevalent in those carrying the risk allele compared to those without (OD 1.74, 95% CI 0.515 TO5.87 and OD 1.50, 95% CI 0.511 to 4.40, respectively).

An interesting observation was made that patients who were heterozygous for the *RasGRP3* mutation appeared to have significantly lower glomerular CD68 cell counts than those without the variant. It is difficult to draw firm conclusions from the data, however, as there was only renal biopsy tissue available from three patients with the risk allele.

4.3.27 FCGR2A (rs1801274) genotyping results

Table 4.51: Association of Fc gamma 2a (rs1801274) polymorphism with lupus nephritis

	European controls (n=379)	European lupus nephritis (n=60)	Allelic p-value	OD	95% CI
GG	25.9% (n=98)	33.3% (n=20)	*0.011	1.67	1.12 to 2.48
AG	48.2% (n=183)	58.3% (n=35)			
AA	25.9% (n=98)	8.3% (n=5)			
	African controls (n=246)	African lupus nephritis (n=45)			
GG	23.2% (n=57)	28.9% (n=13)	0.323	1.25	0.799 to 1.97
AG	51.2% (n=126)	51.1% (n=23)			
AA	25.6% (n=63)	20% (n=9)			
	East Asian controls (n=197)	East Asian lupus nephritis (n=13)			
GG	7.6% (n=15)	23.1% (n=3)	0.121	1.87	0.839 to 4.15
AG	47.7% (n=94)	46.2% (n=6)			
AA	44.7% (n=88)	30.7% (n=4)			

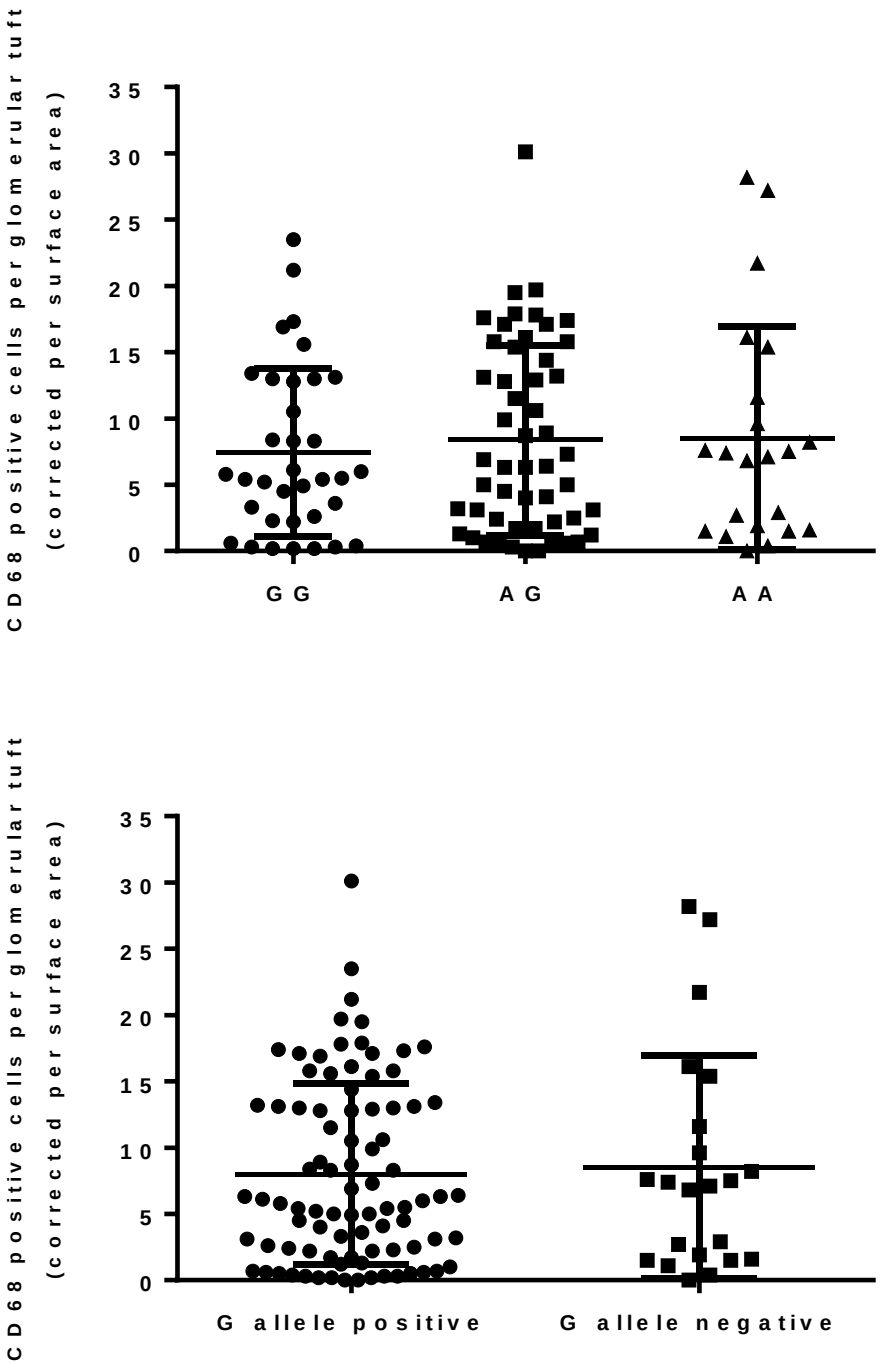
The difference in Fc gamma 2a (rs1801274) allelic distribution between lupus nephritis patients and healthy subjects was examined by Pearson X^2 test and odds ratios (OR) with 95% confidence intervals (CIs) were calculated as an estimation of relative risk for lupus nephritis. A value of $p<0.05$ was considered to be significant. Control data as per 1,000 Genomes Project.

Table 4.52: Univariate analysis exploring clinical parameters associated with the FCGR2A (rs1801274) risk allele

Categorical Variables	Odds ratio	95% Confidence Interval
Gender (female)	0.94	0.291 to 3.01
First degree family history	0.68	0.173 to 2.66
Juvenile onset (<18 years)	0.92	0.351 to 2.38
ESRD	0.68	0.173 to 2.66
ANA	0.40	0.0214 to 7.46
Anti-dsDNA	1.02	0.408 to 2.57
Anti-Ro	1.24	0.493 to 3.12
Anti-RNP	1.13	0.461 to 2.79
Anti-Sm	0.81	0.290 to 2.26
APS	1.21	0.379 to 3.86
Anti-cardiolipin	0.91	0.346 to 2.36
Lupus anticoagulant	0.75	0.303 to 1.84
Continuous Variables	**Mean, SD**	**p-value**
Age at diagnosis (years) Variable present (n=123) Variable absent (n=26)	 26.2 ± 11.6 25.3 ± 10.8	0.957
Duration of follow-up (years) Variable present (n=123) Variable absent (n=26)	 12.3 ± 7.6 9.9 ± 7.4	0.100
Glomerular CD68 count Variable present (n=87) Variable absent (n=22)	 8.0 ± 6.8 8.5 ± 8.4	0.969

None statistically significant

Figure 4.27: CD68 immunostaining in FCGR2A (rs1801274) in proliferative lupus nephritis.



Average number of CD68 positive cells per glomerular tuft (corrected per surface area). There was no significant difference in CD68 positivity between AA, AG and GG genotypes nor was there a difference between those who were A allele positive or negative. Classes I, II and V excluded.

4.3.28 Discussion of FCGR2A genotyping results in lupus nephritis

Fc gamma receptors (FcγRs) are cell surface proteins that recognise the constant (Fc) portion of immunoglobulin. They facilitate antibody-antigen interactions with the immune system and play an essential role in clearance of immune complexes. There are 3 families of FcγR in humans, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). FcγRI is the only high affinity Fcγ receptor while the others are all low affinity. The FcγRs can be defined as either activatory receptors (FcγRIIa, FcγRIIIa, FcγRIIIb) or as an inhibitory receptor (FcγRIIb). The FcγR complex has a high degree of sequence homology and is subject to frequent copy number variation (CNV).

Polymorphisms in FcγRs have been well documented in SLE. Polymorphisms in *FCGR3A* have been associated with SLE and progression to ESRD (Edberg et al., 2002; Magnusson et al., 2004; Alarcon et al., 2006). Low CNV of *FCGR3B* is associated with SLE (Aitman et al., 2006; Willcocks et al., 2008). FcγR polymorphisms have not featured largely in SLE GWAS. This may be due to confounding by high sequence homology and CNV.

We examined the *FCGR2A* rs1801274 polymorphism in our cohort. This polymorphism is caused by a histidine to arginine switch at position 131 (H131R). The R131 and H131 alleles differ substantially in their ability to bind human IgG2 and IgG3. H131 is the high-binding allele while the R131 variant has a lower binding affinity for IgG2, resulting in impaired IgG2-mediated phagocytosis, less efficient immune complex clearance and an increased likelihood of immune complex deposition. Heterozygotes have intermediate IgG2 binding function (Bredius et al., 1993; Shashidharamurthy et al, 2009).

The *FCGR2A* rs1801274 risk variant has been associated with lupus nephritis in studies of European SLE patients (Duits et al 1995; Norsworthy et al., 1999). Other studies have not found an association in Caucasian individuals with SLE (Botto et al., 1996; Manger et al., 1998). The RR genotype has been associated with anti-C1q positivity in lupus nephritis patients (Norsworthy et al., 1999). The R allele has been found to be a significant predictor of lupus nephritis in East Asian SLE populations (Song et al., 1998; Yun et al., 2001). Proteinuria was more severe in those with the R/R rather than the H/R and H/H genotypes. No difference in anti-dsDNA positivity, creatinine clearance or chronicity index was reported in their study (Song et al, 1998). In European patients, the RR genotype was associated with significantly higher levels of proteinuria, haemolytic anaemia, anti-RNP antibodies and low complement levels (Manger et al., 1998). Associations have been reported with lupus nephritis in African American and Hispanic patients (Salmon et al 1996; Zuniga et al., 2001). The *FCGR2A* rs1801274 risk allele has also been linked to Antiphospholipid syndrome (Karassa et al., 2003).

We found an association with the *FCGR2A* rs1801274 risk variant and lupus nephritis in patients of European ancestry. There was no association with familial disease, juvenile onset or increased progression to ESRD. Anti-Ro and anti-RNP antibodies were more common in patients carrying the minor allele (OD 1.24, 95% CI 0.49 to 3.12) (OD 1.13, 95% CI 0.46 to 2.79). Antiphospholipid syndrome was more common in those with the risk allele (OD 1.21, 95% CI 0.38 to 3.86).

4.3 Overall Chapter Conclusion

Using ImmunoChip as a genotyping tool and comparing with ancestral matched control data from the 1,000 Genomes Project, we have found associations in European lupus nephritis patients for both of the HLA region polymorphisms studied, the *IRF5* rs2070197 polymorphism, three *ITGAM* variants, both *STAT4* polymorphisms examined, both *TNFAIP3* variants and in *TNFSF4*. Associations were found in African lupus nephritis patients in *ITGAM* rs1143679, *STAT4* rs7574865 and *ETS1*. All of these polymorphisms have been associated with SLE with genome-wide significance, so it is not surprising that we have found associations with lupus nephritis, even within the confines of our small multi-ancestral cohort. No associations were seen with *IRF7*, *IRF8*, *IRAK1*, *PTPN22*, *NCF2*, *IFIH1*, *BANK1*, *BLK*, *LYN* or *RASGRP3*, however lack of power in this study cannot rule out an association of these susceptibility genes with lupus nephritis.

Some interesting patterns were observed when analysing clinical parameters with the presence of risk alleles in susceptibility genes, particularly regarding age at diagnosis and familial disease. HLA region and *IRF5* polymorphisms are the most strongly associated variants with SLE in GWAS. Risk alleles from both *IRF5* polymorphisms studied and the HLA rs3135394 variant were more likely to be present in adult onset, sporadic cases, predominantly of European ancestry. However the HLA rs9271366 polymorphism was prevalent in familial, juvenile onset cases, as the risk allele was frequently present in African patients.

The *PTPN22* rs247660 polymorphism is one of the most strongly associated genetic risk factors for autoimmunity outside of the HLA region. We found this variant to be almost exclusively present in European lupus nephritis patients, more prevalent in juvenile onset cases but not associated with familial disease.

Genes involved in NFκB pathways were frequently mutated in familial lupus nephritis. The *IRAK1* risk variant was prevalent in juvenile onset, familial cases, and those who progressed to ESRD. Both *TNFAIP3* polymorphisms studied were prevalent in familial disease. Furthermore, a rare variant in *IRAK1* was identified in familial lupus nephritis by WES, which will be discussed later in this thesis.

Genes involved in B-cell receptor signalling studied in our cohort included *BLK, BANK1* and *LYN*. The *BLK* and *BANK1* variants was more prevalent in adult-onset, sporadic disease while the *LYN* polymorphism was frequently seen in familial disease undoubtedly due to the high frequency of the risk allele in African patients.

For the vast majority of polymorphisms examined here, glomerular CD68 count did not differ significantly whether a patient was carrying a risk allele or not. There were, however, some notable exceptions. Individuals who were homozygous for the risk allele in any of the three *ITGAM* polymorphisms studied tended to have low CD68 glomerular counts. Patients with *ITGAM* risk variants tended to be of adult-onset and have sporadic disease. Given the known impairment of phagocytosis and adhesion in those with the *ITGAM* rs1143679 minor allele (MacPherson et al., 2011; Rhodes et al., 2012), it is interesting that glomerular CD68 positive cells were less abundant in patients carrying this variant.

No patients in our cohort were homozygous for the *RASGRP3* risk allele. Seventeen patients were heterozygous for the risk variant and renal tissue was available from ten of these. Three *RASGRP3* heterozygotes had proliferative nephritis on biopsy and were included in our glomerular CD68 analysis. Six *RASGRP3* heterozygotes had class V nephritis and one patient had class II nephritis. *RASGRP3* has also been proposed to have a functional role in phagocytosis in macrophages (Botelho et al., 2009) and it is intriguing that patients carrying risk alleles in these genes would have

predominantly membranous nephritis and a low glomerular CD68 in those with proliferative nephritis.

Two susceptibility genes were associated with higher glomerular CD68 counts, *IKZF1* and those homozygous for the HLA rs9271366 risk allele. Patients with either of these risk variants tended to have juvenile onset nephritis.

The factors that determine glomerular CD68 infiltration remain unclear. Age at disease onset and the influence of genetic variants may certainly play a role. It may be interesting to examine lupus nephritis patients with extremes of glomerular CD68 immunostaining further, by returning to ImmunoChip and testing if other polymorphisms are associated with either end of the CD68 immunostaining spectrum other than the specific polymorphisms that we have already tested.

Chapter 5

Polygenic risk score of lupus susceptibility genes in lupus nephritis patients

The purpose of this chapter is to:

1. Introduce the concept of a polygenic risk score of susceptibility alleles.
2. Assess if a composite genetic risk score of risk alleles is informative regarding the heritability of lupus nephritis in our study group.

5.1 Introduction

Given the modest effects contributed by polymorphisms in individual susceptibility genes to the overall heritability of SLE, an interesting approach would be to combine risk alleles in a composite score for clinical comparison within our lupus nephritis cohort. This approach may help answer some important questions regarding SLE aetiopathology. For example:

- Does age of disease onset correlate with polygenic risk score, considering that younger onset patients have a more severe disease phenotype?
- Can we expect male lupus nephritis patients who generally have earlier onset disease to have a higher polygenic risk score than females?
- Do familial lupus nephritis cases have a higher polygenic risk score than in sporadic onset disease, in that they have inherited an accumulation of susceptibility alleles in common or alternatively do familial nephritis patients have a lower polygenic risk score than sporadic cases pointing towards an alternative monogenic aetiology in these individuals.

As described previously with regard to population stratification, allelic frequency differs significantly between ancestral groups and hence clinical comparisons using polygenic risk score were carried out ancestry by ancestry. When compiling a list of polymorphisms to include in a polygenic risk score it is important to avoid adding multiple SNPs that are in linkage disequilibrium with each which would lead to falsely high or indeed falsely low scores. In addition, it may be preferable to select

SNPs that have been associated with SLE in at least 2 different ancestral groups. By adopting this approach certain genes were eliminated from the composite score in our study, for example *RASGRP3* as it has only been associated with SLE in East Asian individuals. Overall we included 20 SNPs from 17 lupus susceptibility genes in our polygenic score. Genotyping results for these SNPS were obtained from ImmunoChip analysis as outlined in Chapter 4.

5.2 Polygenic risk score computation

Polygenic risk score was assessed by 2 methods, a simple polygenic risk score and a weighted polygenic risk score. The simple polygenic risk score was calculated by counting the number of risk allele carried by an individual (GRS, count GRS).

In the weighted polygenic risk score (wGRS), the risk allele is weighted by the logarithmic odds ratio (log OD) for that allele. The overall wGRS is the sum of the log OD for all risk alleles included in the score divided by the number of variables.

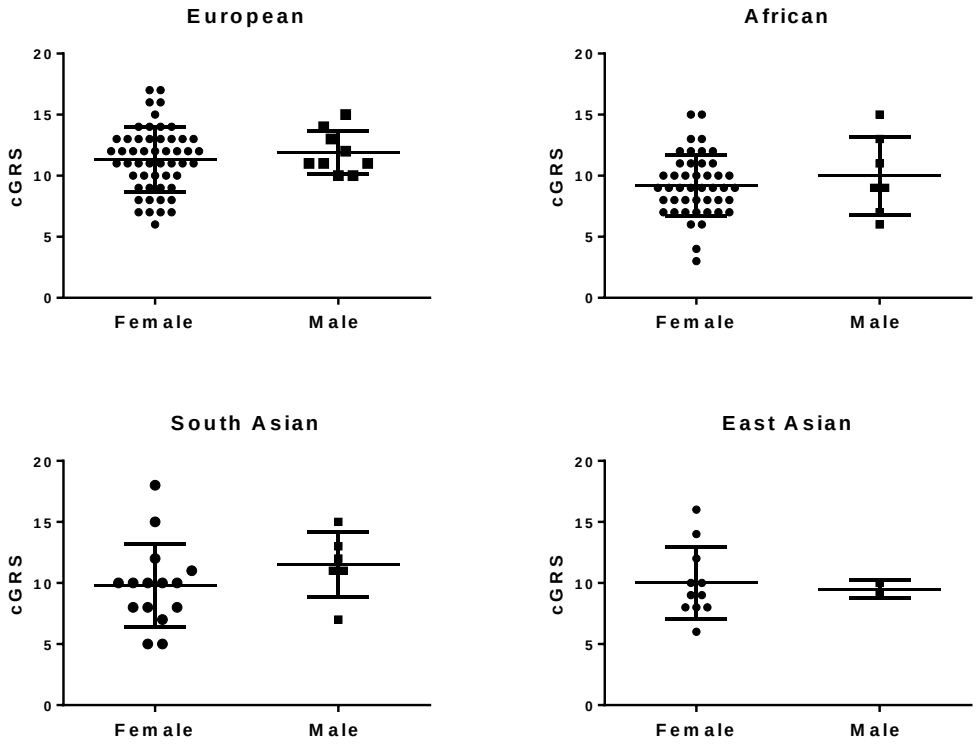
Table 5.1: Lupus susceptibility polymorphisms included in polygenic risk score

Gene	SNPs
HLA region	rs3135394, rs9271366
IRF5	rs2070197, rs10954213
IRAK1	rs2269368
PTPN22	rs2476601
ITGAM	rs1143679
IRF7	rs4963128
IRF8	rs2280381
NCF2	rs10911363
STAT4	rs7574865
IKZF1	rs4917014
IFIH1	rs1990760
TNFAIP3	rs6920220, rs5029939
TNFSF4	rs2205960
ETS1	rs6590330
BLK	rs2736340
BANK1	rs10516487
LYN	rs7829816

5.3 Results

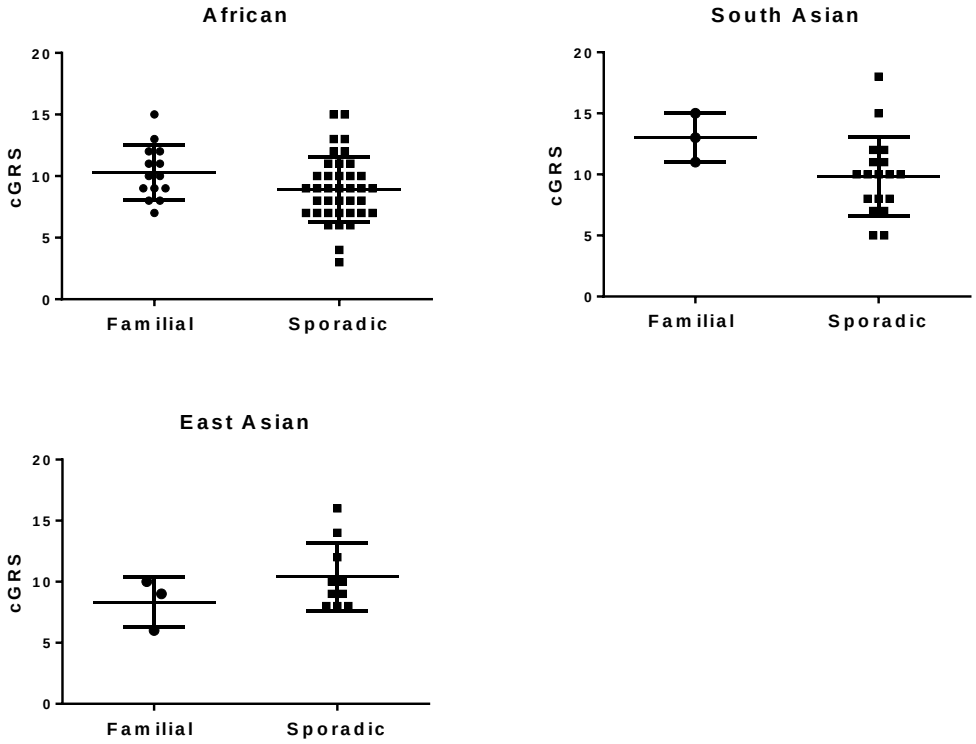
5.3.1 cGRS results

Figure 5.1: cGRS in female versus male lupus nephritis



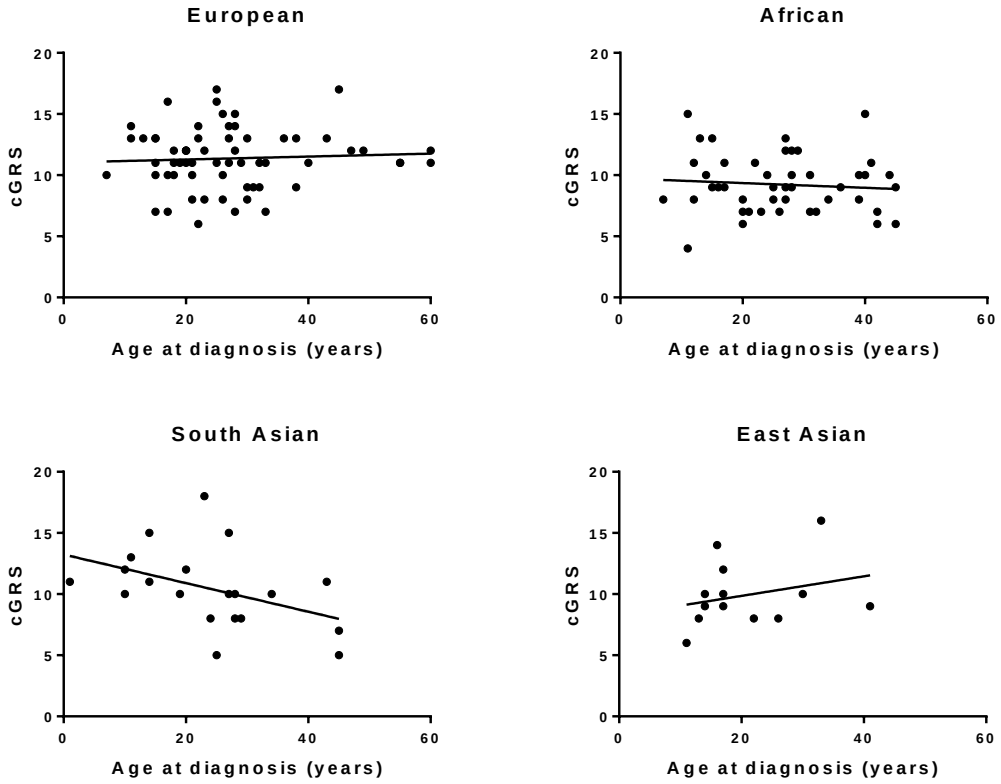
In European lupus nephritis patients the mean cGRS in females was 11.3 ± 2.6 as compared to 11.9 ± 1.8 in males (p=0.542). In African patients, the female cGRS score was 9.2 ± 2.5 and 10.0 ± 3.2 (p=0.637). South Asian female patients had a mean cGRS of 9.8 ± 2.4 and males had a mean score of 11.5 ± 2.7 (p=0.132). East Asian female cGRS was 10.0 ± 2.9 and male was 9.5 ± 0.7 (p=0.949).

Figure 5.2: cGRS in familial versus sporadic lupus nephritis



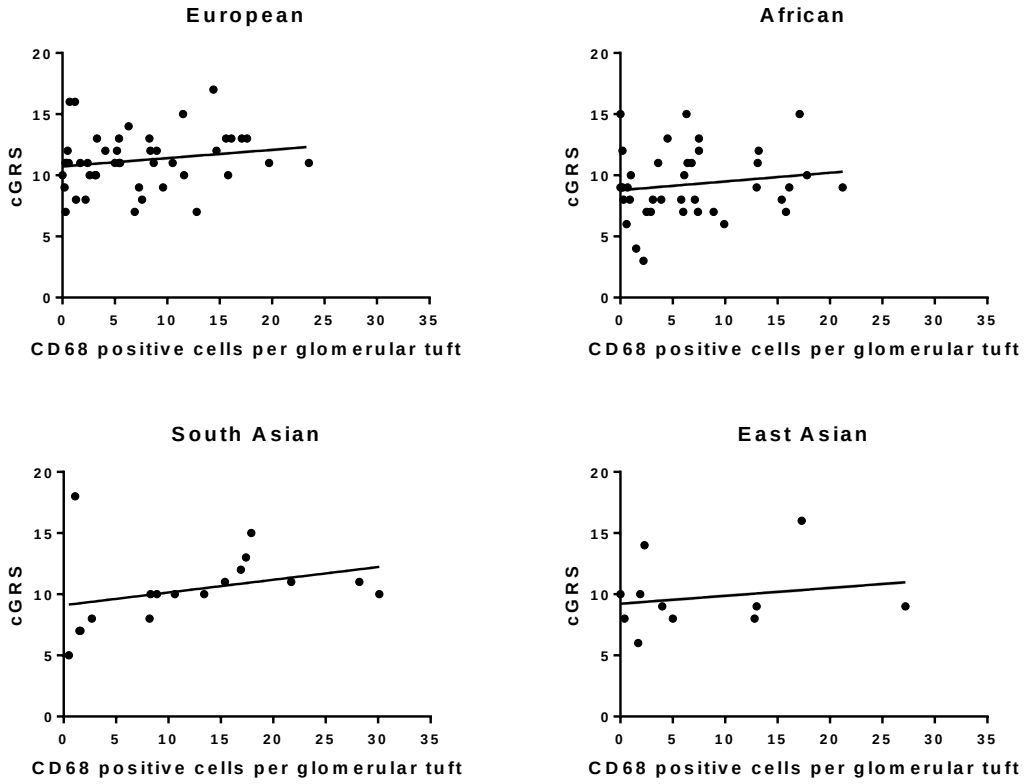
The mean cGRS in African familial cases was 10.3 ± 2.2 in comparison to 8.9 ± 2.7 in sporadic onset African patients (p=0.098). There were no familial patients of European ancestry. South Asian familial patients mean cGRS was 13.0 ± 2.0 while the sporadic patients mean score was 9.8 ± 3.2 (p=0.057). East Asian familial mean cGRS was 8.3 ± 2.1 and 10.4 ± 2.8 in sporadic onset patients (p=0.465).

Figure 5.3: Correlation of cGRS and age at diagnosis



Age at diagnosis of nephritis did not correlate strongly with cGRS in European, African or East Asian patients (r= 0.0581, *p*=0.651) (r= -0.0859, *p*=0.566) and (r=0.269, *p*= 0.375) respectively. South Asian patients with younger onset disease had a higher cGRS (r= -0.433, *p*=0.0567).

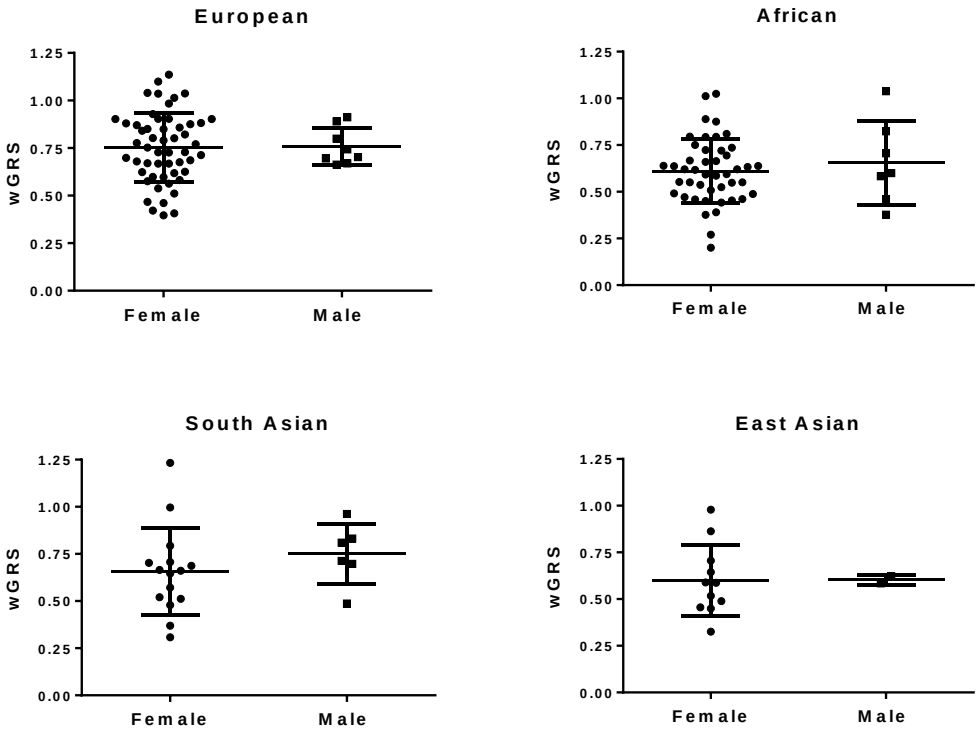
Figure 5.4: Correlation of cGRS and CD68



There were weak positive correlations between cGRS and CD68 count in the glomerular tuft in all ancestral groups; European (r=0.179, *p*=0.238), African (r=0.150, *p*=0.369), South Asian (r=0.312, *p*=0.223) and East Asian (r=0.196, *p*=0.564).

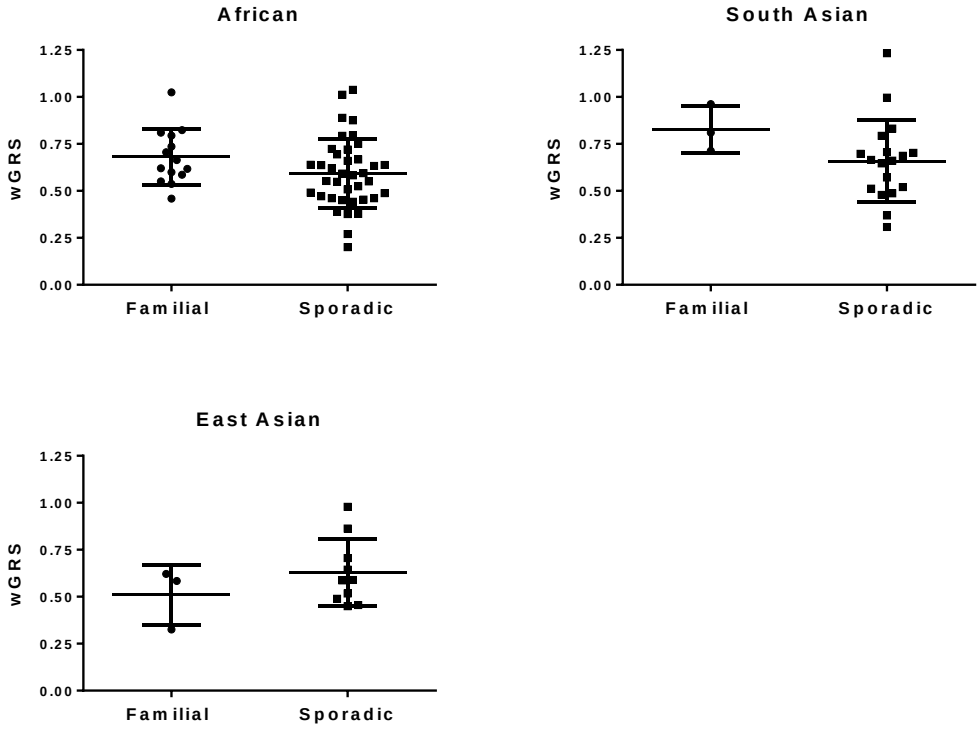
5.3.2 wGRS results

Figure 5.5: wGRS in female versus male lupus nephritis



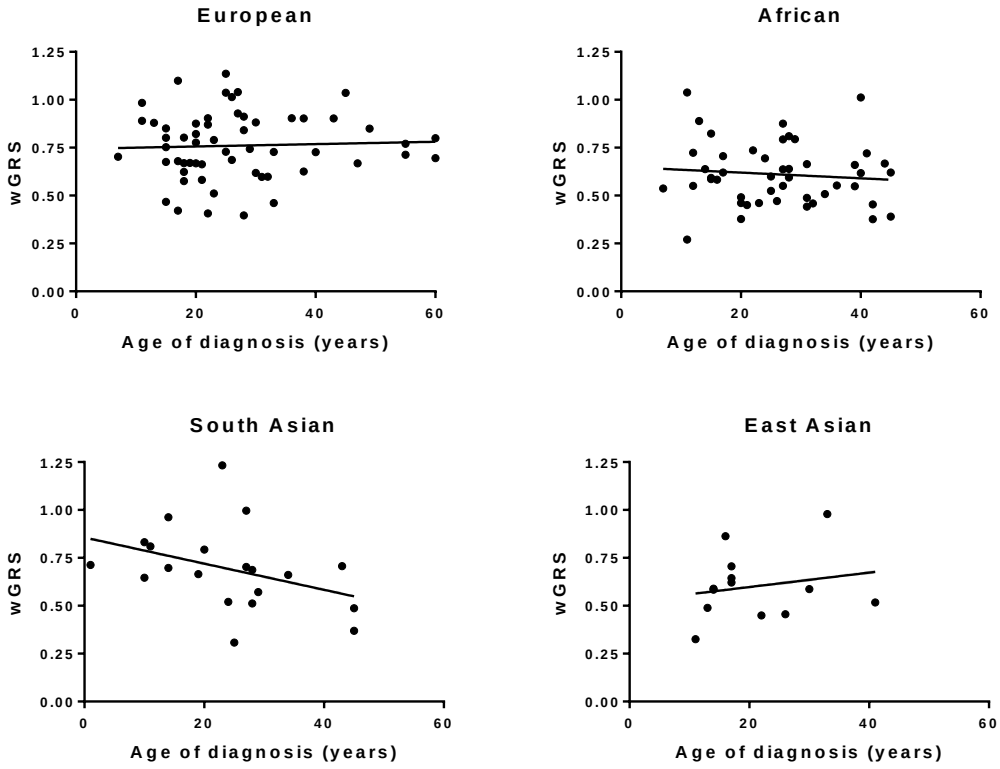
Mean wGRS in female lupus nephritis patients of European ancestry was 0.75 ± 0.18 as compared to 0.76 ± 0.09 in European males (p=0.931). In African patients mean wGRS was 0.61 ± 0.17 in females and 0.66 ± 0.22 in males (p=0.716). South Asian female and male mean wGRS were 0.66 ± 0.23 and 0.75 ± 0.16 respectively (p=0.154). In East Asian patients wGRS was 0.60 ± 0.19 in females and 0.60 ± 0.03 in males (p=0.923).

Figure 5.6: wGRS in familial versus sporadic lupus nephritis



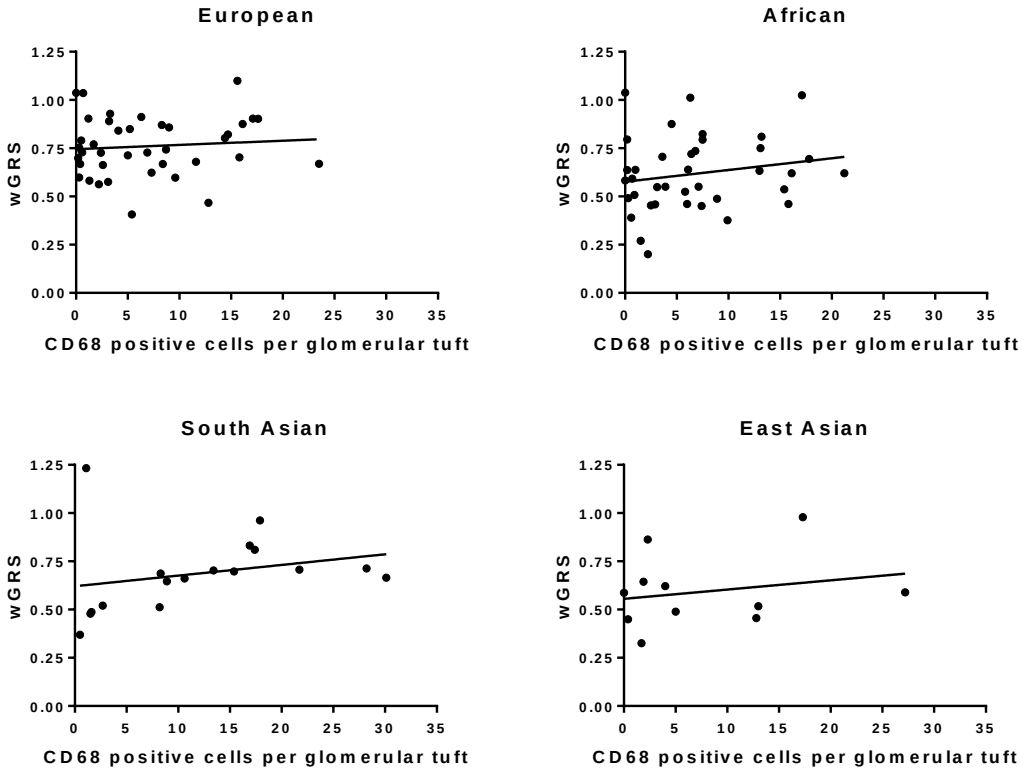
Mean wGRS in African familial cases was 0.68 ± 0.15 as compared to 0.59 ± 0.18 in sporadic onset patients (p=0.121). South Asian familial patients has a mean wGRS of 0.83 ± 0.13 while sporadic onset patients of this ancestry scored 0.66 ± 0.22 (p=0.215). There were no European lupus nephritis patients with familial disease. East Asian familial cases scored 0.51 ± 0.16 in comparison to 0.63 ± 0.18 in sporadic disease (p=0.326).

Figure 5.7: Correlation of wGRS with age of diagnosis



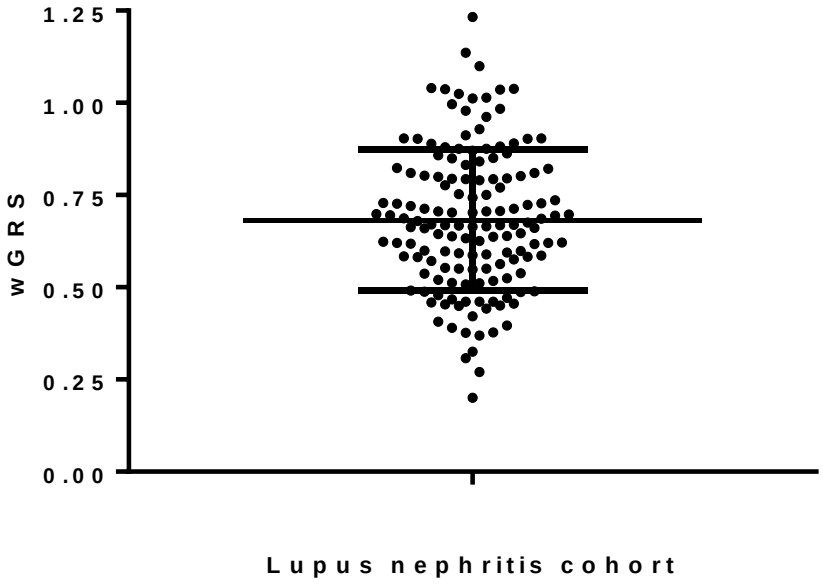
There was no significant correlation between age at diagnosis of lupus nephritis and wGRS in European and African patients, (r=0.044, *p*=0.745) and (r= -0.097, *p*=0.519) respectively. South Asian patients with younger onset disease had a higher wGRS (r= -0.383, *p*=0.097). A weak positive correlation was seen in East Asian patients (r=0.196, *p*=0.521).

Figure 5.8: Correlation of wGRS and CD68



There were weak positive correlations between wGRS and CD68 count in the glomerular tuft in all ancestral groups; European (r=0.0896, *p*=0.588), African (r=0.187, *p*=0.261), South Asian (r=0.253, *p*=0.327) and East Asian (r=0.223, *p*=0.510).

Figure 5.9: Individuals with high and low wGRS



Overall distribution of wGRS in lupus nephritis patients within our cohort, mean wGRS 0.68 ± 0.19 (range 0.20 – 1.23).

5.4 Discussion of relevance of polygenic risk score in lupus nephritis

Overall there was minimal difference in the outcomes of our analysis whether cGRS or wGRS were used, presumably due to the modest odds ratios of most lupus susceptibility alleles.

European lupus nephritis patients in general had the highest cGRS and wGRS, while African and East Asian patients tended to have the lowest scores which validates our approach of analysing each ancestry separately. This phenomenon is presumably due to the fact that most of the SNPs included in the score have stemmed from associations found in GWAS of European SLE patients and to lesser degree East Asian patients and very few large scale studies of African SLE patients exist.

European and East Asian males and females had similar GRS by either scoring system. In African and South Asian patients, there was a minor trend towards males having higher cGRS and wGRS but these did not reach statistical significance.

A trend was seen towards higher cGRS and wGRS in African and South Asian familial cases as compared to sporadic disease but these did not reach statistical significance. The opposite was seen in East Asian lupus nephritis patients where sporadic cases had higher cGRS and wGRS than familial disease.

No convincing correlations were seen between age at diagnosis of lupus nephritis and cGRS or wGRS in European, African or East Asian patients. In the South Asian cohort higher cGRS and wGRS were seen in younger patients. Weak positive correlations between polygenic risk score and CD68 count in the glomerular tuft were seen in all ancestral groups which may be an indicator of disease severity.

A previous study of 1,317 SLE patients comprised of 43% Europeans, 27% African Africans, 12% Hispanic, 10% Gullah, and 2% East Asian patients using a polygenic risk score of 19 SNPs found a significant difference in the number of risk alleles carried by juvenile onset as compared to adult onset patients in Gullah and African American groups. There was no association between the number of risk alleles carried and age of onset in European or Hispanic patients (Webb et al., 2011).

Another study of 1,919 SLE patients all of European ancestry and using a weighted composite score of 22 susceptibility polymorphisms attempted to subphenotype patients and found that age at diagnosis, anti-dsDNA antibodies and haematologic disorder associated with cumulative genetic risk score while nephritis and arthritis were most associated with single known genes in the HLA region and *ITGAM* (Taylor et al., 2011).

It may be interesting to consider the lupus nephritis patients within our cohort who have a low polygenic risk score. Of 24 patients whose wGRS was in the 25th percentile, 14 were African, 4 were East Asian, 3 were South Asian and 3 were of European ancestry. These individuals were generally of adult onset with sporadic disease and may represent suitable candidates for exome sequencing, seeking an alternative genetic aetiology of their disease than the known lupus susceptibility polymorphisms.

Chapter 6

Whole exome sequencing of familial lupus nephritis (Mendelian approach)

The purpose of this chapter is to:

1. Introduce whole exome sequencing (WES) and its applications to Mendelian and complex diseases.
2. Provide an overview of the workflow for WES and functional stratification of gene candidates.
3. Describe the clinical characteristics of 8 families with clustering of lupus nephritis.
4. Highlight the shortlist of gene candidates under consideration for future functional testing.

6.1 Introduction

One of the major areas of interest in SLE research is identifying the causal genes underlying the disease. Variants identified in GWAS and linkage-studies are thought to account for an estimated 15% of the heritability of SLE. In general, common variants identified by GWAS explain a limited amount of the heritability of complex diseases either on an individual basis or when the effects of these variants are combined (Manolio et al., 2009). The genetic heritability that is unknown and remains unexplained is known as 'missing heritability' and may be due at least in part to rare variants with large effect sizes as opposed to the common susceptibility variants with small effect sizes.

WES has been successfully employed to identify both the causal genes underlying Mendelian disorders and to ascertain the extent to which rare variants contribute to the heritability of complex diseases. WES specifically sequences the exons or protein-coding sections of the genome with approximately 5% of the sequencing required for whole genome sequencing (WGS). The first major breakthrough using WES to identify the cause of a Mendelian disorder was the elucidation of the genetic mutations underlying Miller syndrome, a severe disease with prominent facial and limb abnormalities (Ng et al., 2010). Since then, successful application of WES has led to the discovery of the causative variants underpinning multiple diseases.

Rare highly penetrant variants with large effect sizes are of considerable interest as they provide valuable insights into the pathogenesis of disease. Instances of such variants in lupus include mutations in *TREX1*, *DNASE1, DNASE1L3, PRKCD* and in complement pathways (Yasutomo et al., 2001; Crow et al., 2006; Al-Mayouf et al., 2011; Rice et al., 2013; Belot et al., 2013; Rice et al., 2014). These have been discussed in detail in the 'Genetics of SLE' section of the thesis introduction. While

these rare variants do not explain lupus in every single individual with the disease, they have taught us valuable lessons regarding the role of type-I interferon, nuclease defects and immune complex clearance in disease pathogenesis. Each rare variant we discover contributes a further piece of information to advance of understanding of SLE.

Rare variants are unlikely to become significantly frequent in the general population as they are likely to be removed by natural selection due to their detrimental effects on survival. Dominant variants may arise however, as a result of *de novo* mutations and recessive variants may persist at low frequencies in the population. The majority of rare variants with detrimental effects on protein-coding regions of the genome are likely to be of recent origin and tend to be population-specific rather than trans-ethnic (Tennessen, et al., 2012). Novel variants are up to three times more common in individuals of African ancestry than in European or Asian populations (Abecasis et al., 2012).

Careful selection of cases and controls, for example selecting patients with a strong family history of disease will increase the probability of discovering highly penetrant variants with large effects. Unlike common variants with small effects identified by GWAS that are present in the healthy population, variants with large effects are much less likely to be present in unaffected individuals. Thus selecting patients at either end of the phenotypic distribution in an 'extreme phenotype' approach or a 'family based approach' may optimise the chances of discovering causal variants. The 'family-based approach' to WES involves sequencing multiple affected members within the same family and using family pedigree information to direct analysis. Family 1 in our cohort, for example, looks most likely to be an autosomal

recessive pattern of inheritance and hence seeking mutations that are homozygous in the children (probands) and heterozygous in the unaffected parents may be a sensible approach. The 'extreme phenotype' approach assumes genetic homogeneity amongst the affected individuals with hence analysis focusing on rare mutations that are common to a group of patients. A third option for WES is sequencing parent-child trios seeking *de novo* mutations in which the child is affected but the parents are healthy.

6.2 Methods

6.2.1 Workflow for WES

WES of our families with clustering of lupus nephritis was performed in collaboration with Professor Jean-Laurent Casanova, St Giles Laboratory of Human Genetics of Infectious Disease, the Rockefeller University, New York. Analysis of WES data was undertaken by Dr Natasha Jordan during a one-month placement in Professor Casanova's lab with guidance from Dr Bertrand Boisson and Dr Yuval Itan.

Genomic DNA was extracted from thawed whole blood using the GenElute™ Blood Genomic DNA Kit (Sigma Aldrich) as per the manufacturer's instructions. An RNase treatment step was included in the protocol. 5ug of DNA was required with a concentration not less than 40ng/ul for WES. An agarose gel of the DNA samples was run to check for degradation prior to sequencing.

Exome sequencing was carried out at the New York Genomics Centre on an Illumina HiSeq 2000 sequencing machine. Following sequencing, a FASTQ file was produced with all the sequencing reads. BWA (Burrows-Wheeler Aligner) software was then

used to align the sequencing reads against the human genome reference sequence (Li et al., 2009). Further downstream processing was carried out using GATK (genome analysis toolkit), SAMtools (Sequence Alignment/Map) and Picard for removal of duplications, realigning to the genome and variant calling (Li et al., 2009) (McKenna et al., 2010) (http://picard.sourceforge.net). Ultimately, the WES analysis was performed using an annotation software system developed in-house at the Rockefeller University in New York.

6.2.2 *Workflow for WES analysis*

Discrete Filtering

The first step in WES analysis is filtering the variants found in the study patients' data set against known polymorphisms in public databases such as dbSNP, Exome Variant Server (EVS) and the 1,000 Genomes Project (1KG). This narrows down the list of candidates based on the assumption that any variant found frequently in the public filter set cannot be causative. With this in mind and considering the prevalence of lupus nephritis in the general population, we choose mutations where the MAF (minor allele frequency) was <1% for homozygous mutations and where variants were absent from public data bases in the case of heterozygous mutations.

Functional Stratification of Candidates

Following reduction of the number of genes to a minimum number of potential candidates by discrete filtering, candidate genes can be further stratified based on the predicted functional impact or deleteriousness of the mutation in question. Stratification by functional class can be a useful method of prioritising candidates. A stop codon or frameshift mutation, for example, is more likely to have a significant impact on protein-coding than missense mutations.

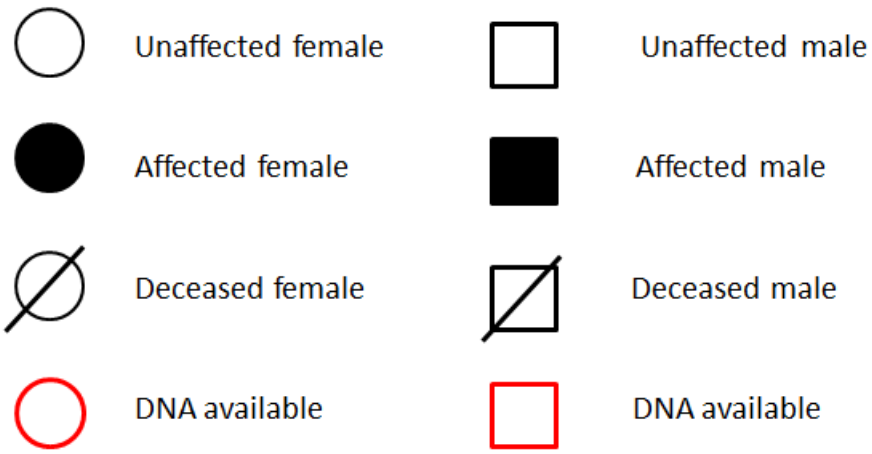
Candidate stratification may be guided by prior knowledge of gene function or its predicted role in a biologic pathway. The candidate gene may be a functional partner of a gene known to be involved in the disease in question. The expression pattern of the gene may provide useful information or a mouse model may yield valuable clues.

Qualitative estimates may provide further evidence of the candidate mutation having a potential functional effect. PolyPhen (Polymorphism Phenotyping) and SIFT (Sorting Intolerant From Tolerant) predict the likelihood of the mutation being damaging based on the predicted change in the protein by amino acid substitutions. PolyPhen is a structure-sequence-based amino acid substitution prediction method and utilizes data available on UniProKB/UniRef100. Its predictions are based on conservation, protein folding and crystal structure (Adzhubei et al., 2010). SIFT predictions are mainly based on sequence homology (Kumar et al., 2009). Neither method is entirely fool-proof with an estimated sensitivity of 68% for PolyPhen and 69% for SIFT**.** Both methods are superior at predicting loss-of-function mutations than gain-of-function variants (Flanagan et al., 2010).

The analysis of our cohort of familial lupus nephritis is outlined as follows:

- Description of the approach to WES analysis for each individual family using pedigree information.
- Due to the large number of variants identified a brief description is provided of a short-list of notable candidates for each family. These candidates include variants of significant functional classes such as stop codons/frameshift mutations, variants predicted to be highly deleterious by PolyPhen/SIFT, and mutations in genes that could be feasibly thought to be involved in the pathogenesis of SLE (Appendices 6-20). The full list of mutations found in each family is available on http://brahma.rockefeller.edu/polyweb
- A more in-depth discussion of candidates being considered for future functional investigation including genes identified with mutations in more than one family of affected patients and genes with functions considered to be highly relevant to lupus pathogenesis. These are described under the following headings:
    - Related groups of genes with candidates in multiple families
    - Individual genes with mutations in multiple families
    - Candidates of interest identified in individual families
- Analysis considering ‘genetic homogeneity’ seeking rare mutation common to the familial lupus nephritis cohort.

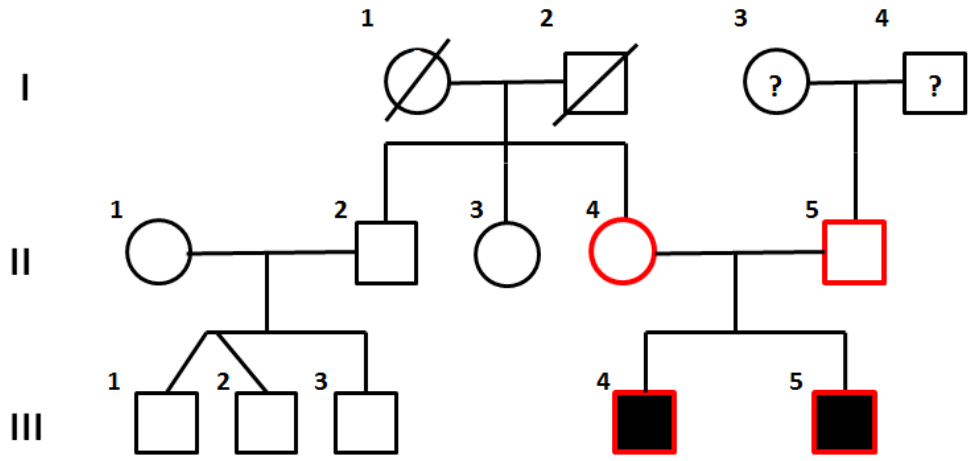
Figure 6.1 Key for family pedigrees



6.3 Results

6.3.1 Clinical vignette of Family 1(Siblings of South Asian ancestry)

Figure 6.2 Family 1 pedigree



The first family with clustering of lupus nephritis identified in our cohort was of two brothers with severe juvenile-onset disease. The parents (II-4 and II-5) are originally from Mauritius of South Asian (India) ancestry and moved to the UK. The probands were born in the UK. No other known relatives had clinical evidence of SLE or autoimmune disease. The father of the family is adopted and hence no clinical information is available on his extended family.

The first brother (III-4) who is currently 29 years old was diagnosed with SLE at 11 years of age with positive ANA, anti-dsDNA antibodies and low complement levels and was initially treated with plasma exchange, methylprednisolone and cyclophosphamide. Repeated renal biopsies showed evidence of diffuse membranoproliferative lupus glomerulonephritis requiring therapy with

mycophenolate mofetil and subsequently with rituximab. In addition, he had evidence of renal thrombotic microangiopathy, positive anti-cardiolipin antibodies and lupus anticoagulant thus fulfilling the classification criteria for APS. Concurrent diagnoses included steroid-induced osteoporosis and hypothyroidism. Despite a severe post renal biopsy haemorrhage leading to nephrectomy and recurrent septicaemia, his renal function remains well preserved with an estimated glomerular filtration rate of 74 (70 - 130 mL/min).

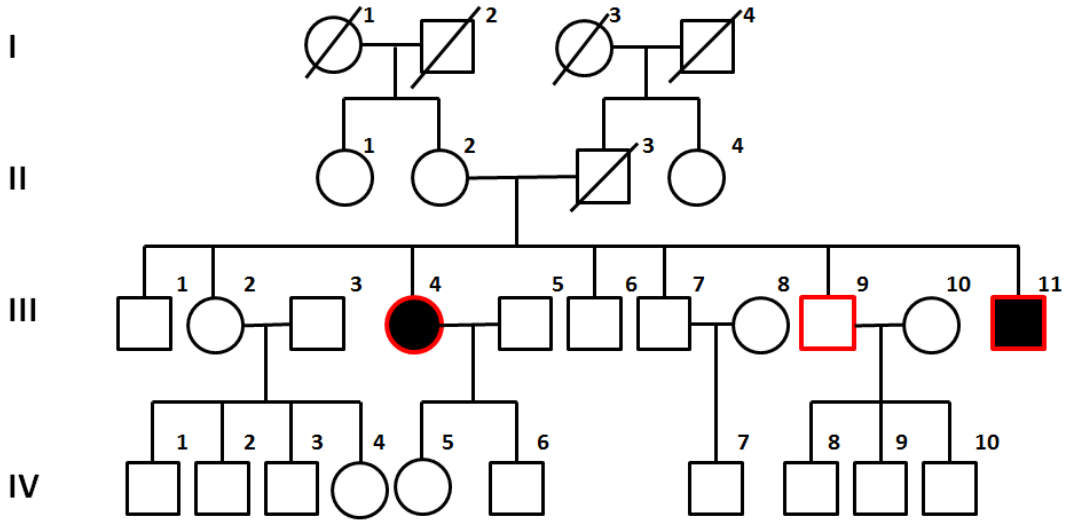
The second brother (III-5) who is currently 26 years old was diagnosed with SLE at age 14 with diffuse proliferative lupus glomerulonephritis on renal biopsy, low complement levels and positive ANA and anti-dsDNA antibodies. Regardless of aggressive immunosuppression including corticosteroids, azathioprine, cyclophosphamide, mycophenolate mofetil and rituximab he developed recurrent episodes of nephritis progressing to end-stage renal failure by age 20. He received a living-related donor renal transplantation from his father at age 21 and despite a number of episodes of acute cellular rejection he remains clinically stable on an immunosuppressive regimen of tacrolimus and sirolimus. WES candidates of interest for Family 1 are summarized in Appendices 6 and 7.

Table 6.1 Approach to WES analysis of Family 1

Genetic Model	Approach	Candidates
Autosomal recessive		
Homozygous	Homozygous mutations in both probands Heterozygous in parents dbSNP/EVS/1KG: MAF<1%	2 candidates
Compound heterozygous	2 heterozygous mutations in same gene in both probands dbSNP/EVS/1KG: MAF<1%	10 candidates
X-linked recessive (mother potential carrier)	Hemizygous in probands Heterozygous in mother dbSNP/EVS/1KG: MAF<1%	4 candidates
De novo mutations	Heterozygous mutations in both probands Not present in parents Not present in dbSNP/EVS/1KG	15 candidates
Autosomal dominant (incomplete penetrance)	Heterozygous mutations in both probands Present in one parent Not present in dbSNP/EVS/1KG	242 candidates Including: 2 stop codons 3 frameshift deletions

6.3.2: Clinical vignette of Family 2 (Siblings of African ancestry)

Figure 6.3: Family 2 pedigree



Family 2 consists of a brother: sister sibling pair with severe lupus nephritis of juvenile onset. Generation II are originally from Nigeria now living in the UK. Generations III and IV were born in the UK. DNA was also available for exome sequencing from an unaffected brother currently aged 33 (III-9). Their other four siblings are unaffected by SLE or other autoimmune diseases. No clinical information was available regarding generation I. Generation II are thought to be unaffected and generation IV are all paediatric and asymptomatic to date.

The sister (III-4) who is currently aged 39 was diagnosed with SLE at age 11 with musculoskeletal involvement and diffuse mesangioproliferative nephritis. She is positive for multiple lupus autoantibodies including ANA, anti-dsDNA, anti-C1q, anti-Sm, anti-RNP and anti-Ro. At the age of 12 she developed neutropenic sepsis which was thought to of iatrogenic origin. During her disease course she has required intensive immunosuppression with cyclophosphamide, azathioprine, methotrexate,

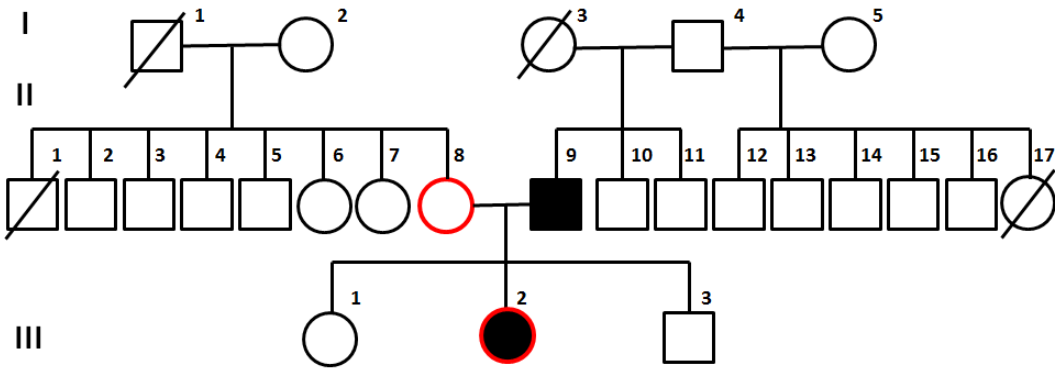
mycophenolate mofetil and corticosteroids for recurrent nephritis. Her most recent renal biopsy in 2009 showed ISN/RPS class IV-S nephritis. At age 32 she had an episode of cryptococcal meningitis. She has also had a suspected episode of septic arthritis of the knee. Currently she remains clinically stable on baseline immunosuppression of azathioprine and low dose corticosteroid. Her renal function is severely impaired with an estimated glomerular filtration rate of 25 mL/min. The brother (III-11) who is currently aged 29 developed SLE with nephritis at disease onset at age 17. He is positive for ANA and anti-Ro autoantibodies. Repeated renal biopsies showed proliferative lupus nephritis and he responded poorly to immunosuppression progressing rapidly to end-stage renal disease. By age 22 he required haemodialysis and underwent renal transplantation by age 25. His lupus activity has remained quiescent since transplantation. WES candidates of interest for Family 2 are summarized in Appendix 8.

Table 6.2 Approach to WES analysis of Family 2

Autosomal recessive Homozygous	Homozygous mutations in both probands dbSNP/EVS/1KG: MAF<1%	1 candidate
Compound heterozygous	2 heterozygous mutations in same gene in both probands dbSNP/EVS/1KG: MAF<1%	31 candidates
Autosomal dominant	Heterozygous mutations in probands Not present in unaffected brother Not present in dbSNP/EVS/1KG	37 candidates

6.3.3 Clinical vignette of Family 3 (Father and daughter of African ancestry)

Figure 6.4: Family 3 pedigree



Family 3 consists of a father: daughter with SLE. Both parents (II-8 and II-9) were born in Nigeria and their children (III-1, III-2 and III-3) were born in the UK. The affected daughter (III-2) who is currently 19 years old was diagnosed with SLE at age 9 with widespread joint involvement and haematologic abnormalities. She developed ISN/RPS class III lupus nephritis at age 10. She is ANA, anti-dsDNA, anti-Ro, anti-RNP and anti-Sm antibody positive. Her disease has proven difficult to control requiring a course of rituximab infusions on two separate occasions. Neither of her siblings, aged 22 and 12 respectively, has developed SLE to date.

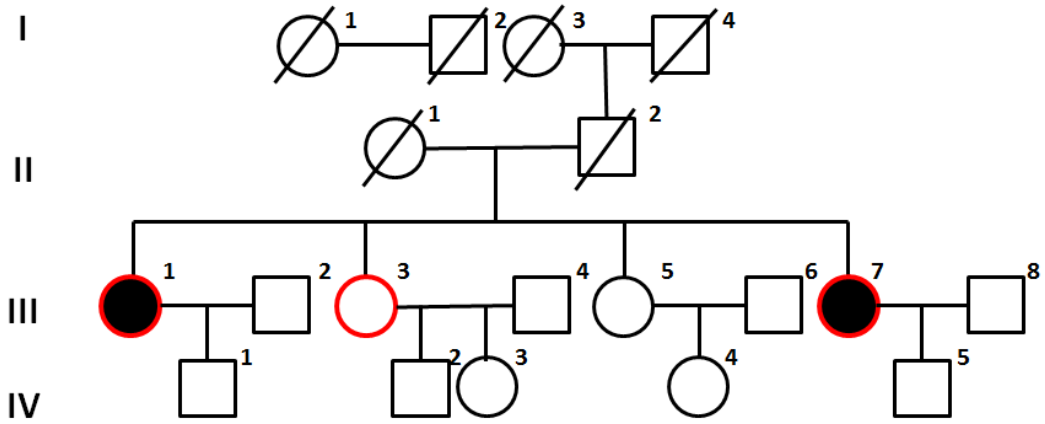
Her father (II-9) who is currently ages 59 was initially diagnosed with discoid lupus in his thirties and subsequently developed ISN/RPS class V lupus nephritis. Unfortunately he has been lost to clinical follow-up and DNA was not available for exome sequencing. WES candidates of interest for Family 3 are summarized in Appendices 9 and 10.

Table 6.3 Approach to WES analysis of Family 3

Genetic Model	Approach	Candidates
Autosomal dominant	Heterozygous mutations present in proband Not present in dbSNP/EVS/1KG	254 candidates Including 32 predicted deleterious by PolyPhen & SIFT
X-linked dominant (father affected)	Heterozygous mutations present in proband Not present in dbSNP/EVS/1KG	10 candidates
Autosomal recessive		
Homozygous	Homozygous mutations present in proband MAF<1% in dbSNP/EVS/1KG	29 candidates
Compound heterozygous	2 heterozygous mutations in same gene in proband MAF<1% in dbSNP/EVS/1KG	77 candidates

6.3.4 Clinical vignette of Family 4 (Siblings of African ancestry)

Figure 6.5: Family 4 pedigree



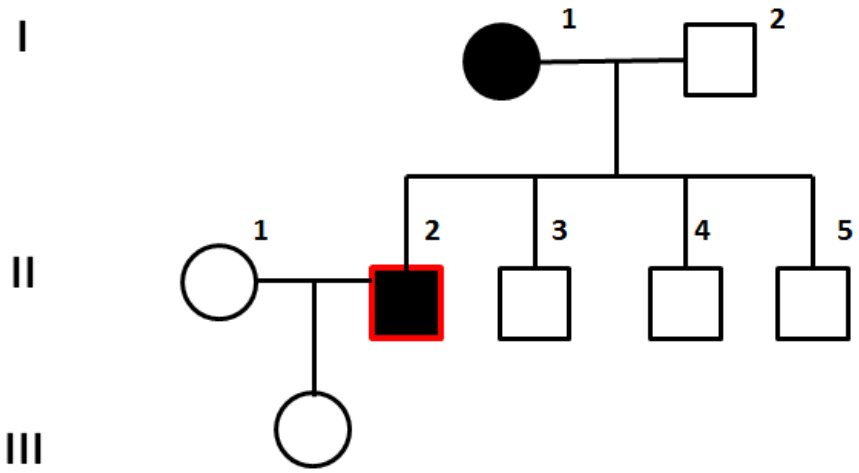
Family 4 is comprised of two sisters with adult onset lupus nephritis. The family is originally from Sierra Leone and now living in the UK. The first sister (III-1) is currently 64 years old and was diagnosed with SLE at age 34 and was found to have WHO class IV nephritis on renal biopsy. She is ANA, anti-dsDNA and anti-Ro antibody positive. The second sister (III-7) is currently 52 years old and was diagnosed with SLE at age 40 also with WHO class IV nephritis on biopsy. She is ANA, anti-dsDNA, lupus anticoagulant and anti -cardiolipin positive. Both sisters have had a relatively benign disease course, responding well to immunosuppression and maintaining their renal function. WES candidates of interest for Family 4 are summarized in Appendices 11 and 12.

Table 6.4 Approach to WES analysis of Family 4

Genetic Model	Approach	Candidates
Autosomal recessive		
Homozygous	Homozygous mutations in both probands dbSNP/EVS/1KG: MAF<1%	5 candidate
Compound heterozygous	2 heterozygous mutations in same gene in both probands dbSNP/EVS/1KG: MAF<1%	33 candidates
Autosomal dominant	Heterozygous mutations in probands Not present in unaffected sister Not present in dbSNP/EVS/1KG	78 candidates

6.3.5 Clinical vignette of Family 5 (Mother and son of South Asian ancestry)

Figure 6.6: Family 5 pedigree



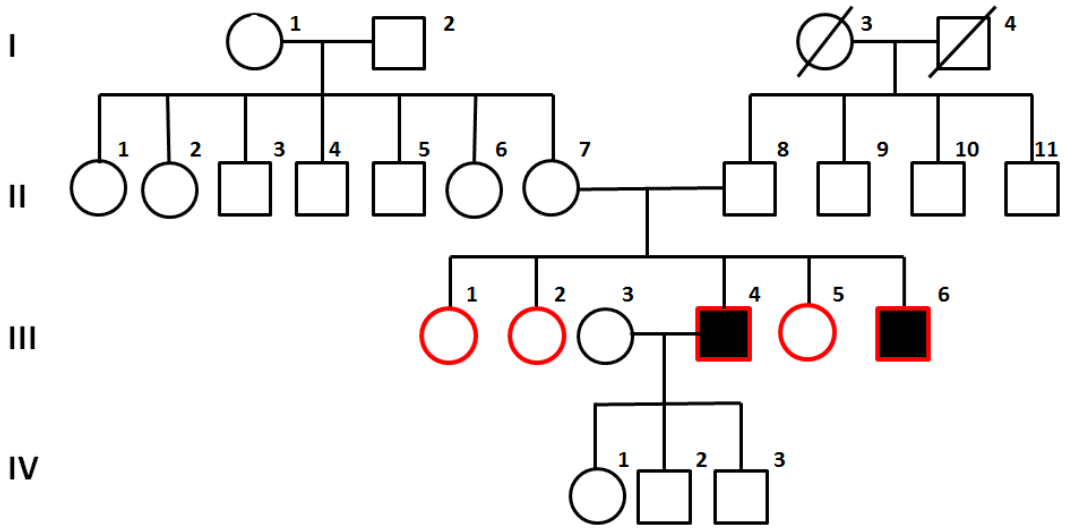
The parents (I-1 and I-2) of family 5 are originally from Kashmir and are now living in the UK. Generations II and III were born in the UK. The affected son (II-2) is the patient with the youngest onset of SLE in our cohort with diagnosis at age 2. He developed recurrent membranous nephritis throughout his childhood and then WHO class IV nephritis in his teens. He is ANA, anti-dsDNA and lupus anticoagulant positive. He progressed to end-stage renal failure and underwent renal transplantation 4 years ago at age 23. His mother (I-1) developed proliferative lupus nephritis in her early 30's when her affected son was 10 years old. Three younger brothers remain unaffected to date. WES candidates of interest for Family 5 are summarized in Appendices 13, 14 and 15.

Table 6.5 Approach to WES analysis of Family 5

Genetic Model	Approach	Candidates
Autosomal recessive		
Homozygous	Homozygous mutations dbSNP/EVS/1KG: MAF<1%	28 candidates
Compound heterozygous	2 heterozygous mutations in same gene dbSNP/EVS/1KG: MAF<1%	26 candidates
X-linked dominant (mother affected)	Hemizygous in probands Heterozygous in mother dbSNP/EVS/1KG: MAF<1%	5 candidates
Autosomal dominant	Heterozygous mutations in proband Not present in dbSNP/EVS/1KG	394 candidates

6.3.6 Clinical vignette of Family 6 (Siblings of East Asian ancestry)

Figure 6.7: Family 6 pedigree



Family 6 consists of 2 brothers with juvenile onset lupus nephritis. The family are originally from Vietnam of Han Chinese ancestry. The parents (II-7 and II-8) were born in Vietnam and moved to the UK. The probands (III-4 and III-6) were born in the UK. They have 3 adult sisters (III-1, III-2 and III-5) who are unaffected.

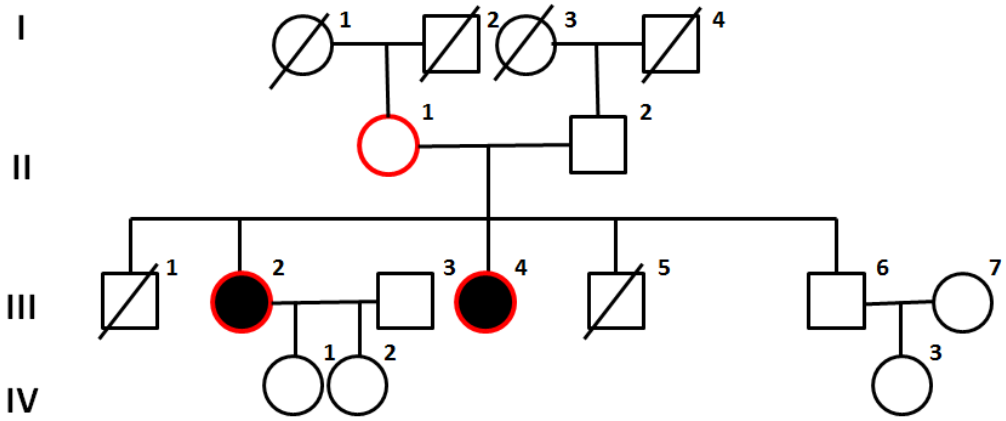
The first brother (III-4) who is now 29 years old was diagnosed with SLE at age 17 with WHO class III lupus nephritis on renal biopsy. 8 years later he had a recurrence of disease and a repeat renal biopsy showed ISN/RPS class III + V nephritis. The second brother (III-6) is currently 27 years old and was diagnosed with SLE at age 14 with WHO class IV nephritis on renal biopsy. A relapse of disease at age 21 necessitated a repeat renal biopsy which showed ISN/RPS class IV-G nephritis. Despite recurrent disease both brothers have maintained their renal function and remain clinically stable on low level maintenance immunosuppression. WES candidates of interest for Family 6 are summarized in Appendices 16 and 17.

Table 6.6 Approach to WES analysis of Family 6

Genetic Model	Approach	Candidates
Autosomal recessive		
Homozygous	Homozygous mutations present in both probands, absent in sisters dbSNP/EVS/1KG: MAF<1%	No candidates
Compound heterozygous	2 heterozygous mutations in same gene dbSNP/EVS/1KG: MAF<1%	10 candidates
X-linked recessive (mother potential carrier)	Hemizygous in probands dbSNP/EVS/1KG: MAF<1%	3 candidates
Autosomal dominant	Heterozygous mutations in proband Not present in dbSNP/EVS/1KG	394 candidates

6.3.7 Clinical vignette of Family 7 (Siblings of African ancestry)

Figure 6.8: Family 7 pedigree



Family 7 consists of two sisters with lupus nephritis. The family is originally from Nigeria; both probands were born in the UK. III-1 and III-5 died in childhood due to Sickle-cell disease. III-2 carries the Sickle-cell trait and III-4 has Sickle-cell disease in addition to SLE.

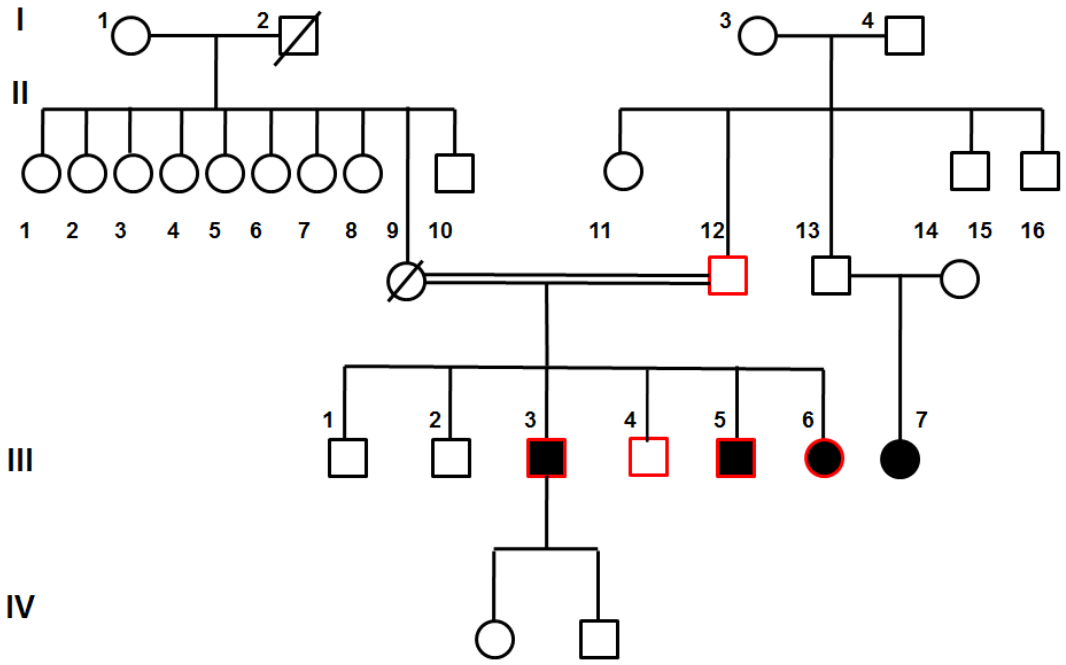
III-2 who is currently 45 years old was diagnosed at age 31 with class II lupus nephritis. She is ANA, anti-dsDNA, anti-Ro, anti-RNP and lupus anticoagulant positive. III-4 who is currently 43 years old was diagnosed at 28 years of age with class V nephritis. She is ANA, anti-dsDNA, anti-RNP and anti-Sm antibody positive. Both sisters remain clinically stable on low dose maintenance immunosuppression and have maintained their renal function. WES candidates of interest for Family 7 are summarized in Appendix 18.

Table 6.7 Approach to WES analysis of Family 7

Genetic Model	Approach	Candidates
Autosomal recessive		
Homozygous	Homozygous mutations in both probands dbSNP/EVS/1KG: MAF<1%	6 candidates
Compound heterozygous	2 heterozygous mutations in same gene in both probands dbSNP/EVS/1KG: MAF<1%	4 candidates
Autosomal dominant (incomplete penetrance)	Heterozygous mutations in both probands Not present in dbSNP/EVS/1KG	36 candidates

6.3.8 Clinical vignette of Family 8 (Siblings of South Asian ancestry)

Figure 6.9: Family 8 pedigree



Family 8 is comprised of 3 siblings (two brothers and a sister) and a female first cousin with SLE. The family is based in Lahore, Pakistan and were identified as part of an ongoing collaboration with the Rheumatology Department there. This is a consanguineous family as the parents (II-9 and II-12) are first cousins. The mother (II-9) has died of breast carcinoma and the father (II-12) is unaffected.

III-3 is currently 33 years old and was diagnosed with lupus nephritis at age 28 years. III-5 has class III lupus nephritis. III-6 is currently 28 years old and was diagnosed with SLE mainly musculoskeletal involvement at age 20. In addition the probands have a first cousin with SLE living in the United States; however the clinical details are not available to us at this time. WES candidates of interest for Family 8 are summarized in Appendices 19 and 20.

Table 6.8 Approach to WES analysis of Family 8

Genetic Model	Approach	Candidates
Autosomal recessive		
Homozygous	Homozygous mutations in all 3 probands dbSNP/EVS/1KG: MAF<1%	8 candidates
Compound heterozygous	2 heterozygous mutations in same gene in all 3 probands dbSNP/EVS/1KG: MAF<1%	2 candidates
Autosomal dominant	Heterozygous mutations in all 3 probands Not present in dbSNP/EVS/1KG	90 candidates

6.4 Discussion of Candidates of Interest from WES for Potential Functional Testing

Related Groups of Genes with Candidates in Multiple Families

6.4.1 Genes involved in activation of Ras pathways

A number of mutations in genes involved in Ras functioning were discovered in our cohort of patients including mutations in *RASGRF2*, *RASSF5* and *RIN1*. Ras proteins are small GTPases that are ubiquitously expressed and play important roles in the modulation of cellular signal transduction. When Ras proteins become activated, they in turn activate other proteins such as those involved in cell growth, differentiation and survival. *HRAS*, *KRAS*, and *NRAS*, the most common Ras genes in humans, are the most frequently mutated oncogenes in human malignancies.

Recently, RASopathies, which include rare neurodevelopmental disorders such as Noonan syndrome and Noonan-related syndromes, have been proposed as novel monogenic conditions predisposing to the development of SLE (Bader-Meunier et al., 2013). A number of genes have been implicated in these syndromes including; *PTPN11, SOS1, KRAS, NRAS, RAF, BRAF* and *SHOC2*. The authors described the case of a 13 year old boy with Noonan syndrome who subsequently developed SLE. Following a literature review they identified 7 further cases of co-existent RASopathy and SLE. These patients tended to have a clinical phenotype including polyarthritis, autoimmune cytopenias and pericarditis but skin involvement and lupus nephritis were not prominent features.

In our study, a heterozygous missense mutation (arginine to cysteine at protein position 640) in *RASGRF2* (Ras protein-specific guanine nucleotide-releasing factor 2) predicted to be highly deleterious by both PolyPhen and SIFT was found in both probands and their unaffected brother in Family 2. *RASGRF2* functions as a calcium-regulated nucleotide exchange factor that activates Ras and RAC1 by exchanging bound GDP for GTP and is involved in the coordination of MAPK pathway signalling (Fan et al., 1998). Interestingly, a predicted functional partner of *RASGRF2, RASGRP3* (Ras guanyl-releasing protein 3) is a known lupus susceptibility gene from an East Asian GWAS. A functional role for *RASGRP3* has been proposed in the activation of Ras and Rap1 during phagocytosis in macrophages (Botelho et al., 2009).

A heterozygous missense mutation (glutamic acid to glutamine at protein position 200) in *RASSF5* (Ras Association (RalGDS/AF-6) domain family member 5) predicted to be damaging by PolyPhen, was found in the proband of Family 5. Synonyms for this gene include *NORE1* and *RAPL*. *RASSF5* is a member of the Ras association domain family and its encoded protein associates with the GTP-activated forms of Ras and Rap1. It has been shown to be an effector molecule in integrin-mediated adhesion and migration of lymphocytes and dendritic cells involving LFA-1 and ICAM-1 (Katagiri et al., 2003, 2004). *RASSF5* may be also be involved in regulation of Ras induced apoptosis (Vos et al., 2003).

A heterozygous missense mutation (arginine to tryptophan at protein position 257) in *RIN1* (Ras and Rab interactor 1), predicted to be deleterious by both PolyPhen and SIFT was found in both probands and absent in an unaffected sister in Family 4. RIN1 is a Ras effector protein that influences Ras signalling on a number of levels. RIN1 interacts with the GTP-bound form of Ras proteins and competes with RAF1

for binding to activated Ras (Han et al., 1997; Wang et al., 2002). RIN1 interacts with the ABL tyrosine kinase enhancing its activity and regulating epithelial cell adhesion and migration (Afar et al., 1997; Hu et al., 2005). In addition, RIN1 activates RAB5A, by exchanging bound GDP for free GTP and facilitates Ras-activated receptor endocytosis (Tall et al., 2001).

6.4.2 Genes involved in the antioxidant system

A recent paper by Ramos et al examined the association of polymorphisms in reactive intermediate genes in SLE patients of different African ancestries. They studied both African American patients who may have some degree of European admixture and a population of Gullah SLE patients. Individuals of a Gullah background are from a genetically homogeneous African group with minimal European admixture. The most significantly associated polymorphism identified in their study was in *GSR* (glutathione reductase) in African American SLE patients. SNPs in *NDUFS4* (NADH dehydrogenase) and *NOS1* (nitric oxide synthase) were most strongly associated with the Gullah patients. When both population groups were combined, the *GSR* polymorphism remained the most significant association overall (Ramos et al., 2013).

In our cohort, rare mutations have been identified in *GSR*, *NOS1* and *NDUFA13*. In Family 5, there was a homozygous mutation in *GSR* for a stop codon at position 32, predicted to be highly deleterious and have significant functional consequences. A further heterozygous mutation in *NDUFA13* (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 13) was seen in Family 5, resulting from an arginine to glutamine substitution at protein position 81, that was also predicted to be highly damaging by PolyPhen and SIFT. A mutation in *NOS1* was discovered in both probands of Family

4, that resulted from a glycine to valine substitution at protein position 50 and was predicted to be highly damaging by both PolyPhen and SIFT.

Overproduction of nitric oxide and reactive oxygen species and an altered redox state are well recognised in SLE patients and has been shown to correlate with disease activity, complement levels, proteinuria, renal damage and lack of response to therapy (Kurien et al., 2003; Oates et al., 2008; Wang et al., 2010). GSR is a central enzyme of cellular antioxidant defence. Its primary role is in the reduction of oxidized glutathione disulfide to glutathione (GSH). There is increasing evidence that depletion of intracellular GSH may play a role in dysregulation of apoptosis (Franco et al., 2009). An early event in the cascade of apoptosis is a decrease in cellular GSH. GSH is essential for cell survival as demonstrated in mouse models with abnormal GSH undergo massive apoptotic cell death (Valverde et al., 2006).

Rare mutations in *GSR* have been reported to result in favism, hyperbilirubinaemia, and cataracts. GSR deficiency in erythrocytes was previously reported in an SLE patient that was correctable *in vivo* with riboflavin. The same deficiency was found in the patient's mother and other relatives who were asymptomatic (Fajnholc et al., 1971). A previous report of a consanguineous family of three children with almost complete absence of GSR activity in erythrocytes resulted in favism in one child and cataracts in the other two. GSR activity in leucocytes was also reduced in this family but no history of recurrent infections or autoimmunity was reported (Loos et al., 1976).

GSR potentiates host defence by sustaining phagocytic oxidative burst and enhancing production of NETs (Yan et al., 2012). This may be of relevance in SLE given the increasing evidence of the role of NETs in lupus pathogenesis (Lande et al., 2011; Garcia-Romo et al., 2011; Villanueva et al., 2011). GSH levels are

decreased in peripheral T-lymphocytes from SLE patients (Gergely et al., 2002; Shah et al., 2010, 2011). (NZB NZW) F1 lupus prone mice treated with N-acetyl cysteine, a precursor of GSH have less autoantibody production, less severe nephritis and increased survival (Suwannaroj et al., 2001).

6.4.3 Synaptotagmin-like proteins (SLPs)

A number of mutations in genes encoding synaptotagmin-like proteins were identified in our cohort. A non-frameshift mutation in *SYTL5* was found in both male probands in Family 6. A heterozygous missense mutation (aspartic acid to histidine at protein position 25) in *SYTL3*, predicted to be highly deleterious by both PolyPhen and SIFT, was found in both male probands in Family 1. A heterozygous intronic mutation in *SYTL2* was found in Family 5.

In addition, two compound heterozygous mutations in *MLPH* (glutamic acid to lysine at protein position 323) and (glutamic acid to valine at protein position 363), both of which were predicted to be highly deleterious were identified in Family 3. *MLPH* encodes the protein for melanophilin and is predicted to be a functional partner of the SYTL genes.

SLP protein family members contain a unique homology domain at the N-terminus, known as the Slp homology domain (SHD). The SHD has been shown to specifically bind to the Ras-related GTPase Rab-27A which has a proposed regulatory role in the function of lysosome-related organelles, including melanosome motility in melanocytes (Strom et al, 2002; Kuroda, 2004).

MLPH encodes a member of the exophilin subfamily of Rab effector proteins. The protein forms a complex with Rab27A and myosin Va (Bahadoran et al., 2003).

Mutations in *MLPH* and *RAB27A* lead to impairment of melanosome transport and result in haemophagocytic syndrome and abnormal hair pigment distribution in Griscelli syndrome (Bahadoran et al, 2003).

6.4.4 Mutations in genes encoding the epsilon DNA polymerase complex

The central component of the epsilon DNA polymerase complex consists of four subunits: POLE, POLE2, POLE3 and POLE4. Mutations in *POLE* and *POLE3* were found in our cohort of patients. *POLE* (polymerase (DNA directed), epsilon, catalytic subunit) encodes the catalytic subunit of DNA polymerase epsilon, an enzyme involved in DNA repair. Mutations in *POLE* have been associated with colorectal cancer and FILS syndrome (facial dysmorphism, immunodeficiency, livedo, and short stature) (Pachlopnik Schmid et al., 2012; Palles et al., 2013). *POLE3* (polymerase (DNA directed), epsilon 3, accessory subunit) is also known as *CHRAC17* (chromatin accessibility complex, 17-KD subunit) and encodes a histone-fold protein.

A heterozygous missense mutation (isoleucine to phenylalanine at protein position 238) in *POLE* predicted to be highly deleterious by PolyPhen and SIFT, was identified in both male probands and two of three unaffected sisters in Family 6. A heterozygous missense mutation (valine to leucine at protein position 69) in *POLE3* predicted to be highly deleterious by PolyPhen and SIFT, was identified in all 3 probands in Family 8. This mutation was absent in their unaffected father and sibling.

6.4.5 Chromodomain helicase DNA binding proteins

Chromodomain helicase DNA binding (CHD) family are ATP-dependent chromatin remodellers. The CHD family are divided into 3 subgroups of genes; *Chd1-Chd2* subfamily, *Chd3-Chd4* subfamily, and *Chd5-Chd9* subfamily. CHD family members have been shown to have roles in transcriptional activation, repression and elongation (Woodage et al., 1997; Kelley et al., 1999). They interact with H3K4me and may have HDAC activity (Flanagan et al., 2005; Pray-Grant et al., 2005). A number of human diseases have been associated with *CHD3* and *CDH4* including Hodgkin's lymphoma and neuroblastoma (Law et al., 2005; White et al., 2005). In addition, *CHD3* and *CHD4* have been identified as autoantigens in the autoimmune condition dermatomyositis (Seelig et al., 1995). CHARGE syndrome, a rare disease with ocular abnormalities, cardiac defects, renal and genital anomalies, growth retardation and deafness has been associated with *CHD7* (Vissers et al., 2004).

In our cohort, mutations were identified in two CHD family members, *CDH8* and *CHD9*. In Family 3, compound heterozygous mutations (glutamic acid to lycine at protein position 88) and (alanine to serine at protein position 1132) were identified in *CHD8* in the proband. Only one of these mutations was present in the unaffected mother. Neither of these mutations were predicted to be deleterious by PolyPhen or SIFT, however.

In Family 8, in which there was a history of consanguinity, all three probands were homozygous for a missense mutation (proline to arginine at protein position 2114) in *CHD9* which was predicted to be highly damaging by both PolyPhen and SIFT. Their unaffected sibling and father were heterozygous for this mutation.

6.4.6 Genes involved in ubiquitination pathways

Ubiquitination is a process by which proteins undergo post-translational modification by the addition of ubiquitin molecules, initiating a chain of events resulting in the degradation of the protein by the proteasome. Polymorphisms in genes involved in ubiquitination have been associated with SLE such as *TNFAIP3, TNIP1* and *UBE2L3*. Mutations in 2 genes involved in ubiquitination were found in our patient cohort; *USP36* and *UBR4*.

Missense mutations in *USP36* (ubiquitin specific peptidase 36) were identified in 2 families within our cohort. In Family 3, a heterozygous missense mutation (valine to leucine at protein position 409) in *USP36* was present in the affected daughter but absent in the unaffected mother and was predicted to be highly deleterious by both PolyPhen and SIFT. In the proband of Family 5, a further *USP36* heterozygous mutation (glycine to valine at protein position 468) was identified and predicted to be moderately deleterious by PolyPhen and highly deleterious by SIFT.

Compound heterozygous mutations (cysteine to phenylalanine at protein position 933) (leucine to valine at protein position 4129) in *UBR4* (ubiquitin protein ligase E3 component n-recognin 4) were found in the proband of Family 5. The former was predicted to be highly deleterious while the later was not.

USP36 is part of a family of cysteine proteases that function as deubiquitination enzymes (Quesada et al., 2004). *UBR4* encodes a protein present in the cytoskeletal component of the cytoplasm and the chromatin scaffold in the nucleus. In combination with clathrin, it creates meshwork structures such as those involved in cytoskeletal organization. *UBR4* may also play a role in regulation of integrin-mediated signalling (Nakatani et al., 2005).

6.4.7 Serine/threonine protein kinases

A number of mutations in serine/threonine protein kinases were identified in our lupus families including *MAPK3, MAP3K6* and *STK33.*

The 3 probands of Family 8 were all discovered to have a homozygous *MAPK3* mutation (glycine to aspartic acid at protein position 9). This was not predicted to be damaging by either qualitative score, however. The unaffected father was heterozygous for this mutation and the unaffected sibling did not have the mutation. A frameshift deletion in *MAP3K6* (mitogen-activated protein kinase kinase kinase 6) was seen in the proband of Family 5.

MAPK1/ERK2 and MAPK3/ERK1 act in a signalling cascade regulating vital cellular processes. Following activation by upstream kinases, they, in turn, become activated, resulting in translocation to the nucleus where nuclear targets are phosphorylated. Defective activity of *MAPK1* and *MAPK3* has been seen in peripheral blood T-lymphocytes of SLE patients and may be linked to altered coupling of Ras guanine nucleotide exchange factor hSos (human Son of Sevenless) to the adapter protein Grb2 (Cedeno et al, 2003).

Mutations in *STK33* (serine/threonine-protein kinase 33) were present in 2 individual families in our cohort. In Family 2, a heterozygous missense mutation (lysine to asparagine at protein position 512) was present in both probands and their unaffected brother and predicted to be highly deleterious. In Family 5, a further heterozygous mutation (isoleucine to threonine at protein position 306) was identified in *STK33* in the proband and was predicted to be moderately deleterious by PolyPhen and highly deleterious by SIFT.

STK33 is a member of the CAMK group (Ca2+/calmodulin-dependent kinase) of serine/threonine kinases. *STK33* was found to be critical for abnormal cell growth in human cell lines expressing mutations in the oncogene *KRAS*. A knock down of *STK33* using siRNA induced apoptosis in *KRAS*-dependent Acute Myeloid Leukaemia cell lines (Scholl et al., 2009). When small-molecule inhibitors of *STK33* were used to target *KRAS*-dependent cells, however, they were found to be ineffective (Luo et al., 2012). Interestingly, polymorphisms in *STK17A* were associated with SLE in a recent Brazilian study (da Silva Fonseca et al., 2013).

6.4.8 Mutations in TLR and interferon signalling pathways

Mutations in *IRF9* (interferon regulatory factor 9) were found in 2 families in our study. In Family 1, a heterozygous missense mutation (arginine to histidine at protein position 102) in *IRF9* was present in the both probands but was not predicted to be deleterious by either PolyPhen or SIFT. In Family 3, compound heterozygous mutations in *IRF9* were present in the proband (valine to phenylalanine at protein position 222) and (alanine to glutamic acid at protein position 258). Neither of these mutations was predicted to be damaging however. A heterozygous mutation in *TLR9* was present in both probands of Family 1 (alanine to serine at protein position 1185) and was predicted to be deleterious by SIFT but not by PolyPhen. Given the known role of endosomal TLRs and type I interferon pathways in lupus pathogenesis as discussed earlier in this thesis, these candidates are certainly of interest for future functional testing.

Individual Genes with Mutations in Multiple Families

6.4.9 MACF1

Mutations in *MACF1* (microtubule-actin crosslinking factor 1) were discovered in three families in our cohort. *MACF1*, also known as *ACF7* (actin cross-linking family protein 7) is a member of a family of proteins whose primary role is the formation of bridges between different cytoskeletal elements (Kodama et al., 2003). *MACF1* also acts as a positive regulator of Wnt signalling pathways (Chen et al., 2006).

In Family 8, compound heterozygous *MACF1* mutations were found in all 3 probands (threonine to asparagine at protein position 166) (cysteine to phenylalanine at protein position 3484). The first predicted to be moderately deleterious by PolyPhen and highly deleterious by SIFT but the second was not predicted to be damaging.

In Family 3, compound heterozygous mutations (arginine to glutamine at protein position 389) (valine to isoleucine at protein position 1692) in *MACF1* were seen in the proband. The former was predicted to be deleterious while the later was not. One of these mutations was present in the unaffected mother in this family.

In Family 5, compound heterozygous *MACF1* mutations (glutamine to histidine at protein position 2980) (arginine to glutamine at protein position 1070) were found in the proband. The first was predicted to be deleterious by both PolyPhen and SIFT while the second was not.

6.4.10 DDR1

Mutations in *DDR1* (discoidin domain receptor tyrosine kinase 1) were identified in 2 separate families in our cohort. In Family 5, a homozygous missense mutation

(arginine to glutamine at protein position 119) was present in the proband and was predicted to be moderately deleterious by PolyPhen but not by SIFT. In Family 3, a heterozygous mutation (glutamine to glutamic acid at protein position 38) was present in both the proband and the unaffected mother but was not predicted to be deleterious.

DDR1 is a cell surface tyrosine kinase receptor. Once activated by binding to fibrillar collagen, it regulates cell differentiation, proliferation and migration (Vogel et al., 1997; Hou et al., 2002). *DDR1* has been proposed to play a role in renal injury. There is increased expression of *DDR1* in the glomeruli of mice and in podocytes and cellular crescents of humans with crescentic glomerulonephritis (2 lupus nephritis cases and 3 Goodpasture's syndrome cases) (Kerroch et al., 2012). Inhibition of *DDR1* activity by deletion of the gene or injection of antisense oligodeoxynucleotides protects against crescentic glomerulonephritis and loss of kidney function in mice (Kerroch et al., 2012).

6.4.11 COL6A5

Mutations in *COL6A5* (collagen, type VI, alpha 5) were found in 2 separate families in our cohort. Compound heterozygous mutations (stop codon at protein position 1563) (threonine to proline at protein position 299) were found in both probands and their unaffected brother in Family 2. A heterozygous missense mutation (phenylalanine to valine at protein position 680) was identified in the proband of Family 5 and predicted to be highly deleterious by PolyPhen but not by SIFT. COL6A5 belongs to the family of collagens containing von Willebrand factor type A domains, which are involved in organisation of tissue architecture and cell adhesion. *COL6A5* is highly expressed in the skin and to a lesser degree in the lung, small

intestine and colon (Soderhall et al., 2007). Variants in *COL6A5* were previously reported to be associated with atopic dermatitis. Subsequent studies, however, have failed to show an association (Soderhall et al., 2007; Naumann et al., 2011).

6.4.12 BOC

Mutations in *BOC* (BOC cell adhesion associated, oncogene regulated) were present in Family 4 and Family 6, although neither was predicted to be damaging. *BOC* is a member of the immunoglobulin/fibronectin type III repeat family. It is a component of a cell-surface receptor complex with CDON (cell adhesion associated, oncogene regulated) that mediates cell-cell interactions and promotes myogenic differentiation (Kang et al., 2002).

Candidates of Interest for Potential Functional Testing Identified in Individual Families

6.4.13 TRAP1/DNASE1

In Family 5, compound heterozygous mutations were found in the exon of *TRAP1* overlapping with the intron of *DNASE1*. The first mutation was an arginine to glutamine substitution at position 650 and was not predicted to be deleterious. The second mutation was a threonine to lysine substitution at position 65 and was also not predicted to be deleterious.

Dnase-1 deficient mice develop clinical features strongly reminiscent of human lupus with autoantibody production and glomerulonephritis (Napirei et al., 2000). A functional heterozygous missense mutation in *DNASE1* was previously identified in 2 unrelated Japanese SLE patients (Yasutomo et al., 2001). An autosomal recessive

mutation in *DNASE1L3* has been described in familial juvenile onset SLE patients in Saudi Arabia (Al-Mayouf et al., 2011).

TRAP1 (tumour necrosis factor receptor associated protein 1) is a member of the heat shock protein 90 family (HSP90). TRAP1 is a mitochondrial chaperone protein whereas most other HSP90 proteins are mainly located in the cytosol and endoplasmic reticulum (Chen et al., 2005). Annotated transcripts from *TRAP1* are known to overlap with transcripts from the *DNASE1* gene suggesting that transcription of one of these genes will strongly influence the other (Fismen et al., 2013).

In the NZBxNZW lupus mouse model, progression from mild to end-stage renal disease corresponds with significant reduction in DNaseI expression. This down regulation of DNaseI leads to decreased chromatin fragmentation and subsequent deposition of these chromatin fragments in the glomerular basement membrane where they form immune complexes with immunoglobulin (Fenton et al., 2009) (Thiyagarajan et al., 2012; Fismen et al., 2013). Trap1 is progressively upregulated during this process as demonstrated by qPCR and Western blot analyses (Fismen et al., 2013). In human lupus nephritis, immunohistochemistry of renal sections shows normal DNASE1 staining in control kidneys and in mesangial lupus nephritis but significantly reduction of staining in ISN/RPS class IV. TRAP1 immunostaining is similar between controls and lupus nephritis biopsies (Fismen et al., 2013).

6.4.14 ADCY7

In Family 8, all 3 probands had a homozygous *ADCY7* mutation (alanine to valine at protein position 480). This mutation was predicted to be moderately deleterious by

PolyPhen and highly damaging by SIFT. Their unaffected father was heterozygous for this mutation while their unaffected sibling did not have the mutation.

Adenylyl Cyclases (ACs) are a group of enzymes that catalyse the formation of cyclic AMP from ATP (Ludwig et al., 2002). *ADCY7* has been implicated in the neuronal regulation of mood and may be involved in the pathophysiology of depression (Joeyen-Waldorf et al., 2012). Polymorphisms in *ADCY7* have been associated with alcohol dependence (Desrivières et al., 2011). *ADCY7* expression is significantly upregulated in abdominal aortic aneurysm tissue (Hinterseher et al., 2013).

6.4.15 DEFB119

Compound heterozygous mutations in *DEFB119* (defensin, beta 119) were found in both probands of Family 6 and only one of these mutations was found in each of their three unaffected sisters. The first mutation was an alanine to valine substitution at protein position 33 which was not predicted to be damaging. The second mutation was a threonine to asparagine substitution at protein position 70 which was predicted to be moderately deleterious by PolyPhen and highly damaging by SIFT.

There are a growing number of reports in the literature supporting the role for defensins in lupus pathogenesis. Defensins are natural antimicrobials and an important component of innate immunity. Interestingly, they are known to contribute to the formation of NETs. α-defensin is a granulopoiesis-related marker and is upregulated alongside the interferon-α signature in SLE patients (Bennett et al., 2003; Ishii et al., 2005; Sthoeger et al., 2009). β-defensin and human neutrophil peptides are elevated in SLE patients and these correlate with disease activity

(Vordenbaumen et al., 2010). Polymorphisms in DEFB1 have been associated with SLE in Brazilian patients (Sandrin-Garcia et al., 2012). CNV of DEFA1A3 has been described in East Asian SLE patients (Cheng et al., 2013).

6.4.16 PTBP3

A homozygous mutation in *PTBP3* (polypyrimidine tract binding protein 3) that resulted from a proline to leucine substitution at position 7 was found in both probands in Family 1. This mutation was predicted to be highly deleterious by both PolyPhen and SIFT. Both parents were heterozygous for this mutation.

PTBP3, also known as ROD1 (regulator of differentiation 1) is an RNA binding protein that regulates differentiation (Yamamoto et al., 1999). *ROD1* may play a role in cell survival during hypoxia (Fasanaro et al, 2012). Knock down of ROD1 by siRNA inhibits motility of lung cancer cells *in vitro* (Tano et al., 2010). There has not been any literature published to date on the role of *PTBP3/ROD1* in SLE or autoimmunity.

6.4.17 TRAK1

A non-frameshift deletion in *TRAK1* (trafficking protein, kinesin binding 1) was found in the probands of Family 1. *TRAK1* has been shown to regulate endocytic trafficking of GABA receptors in the central nervous system and mutations in mice result in hypertonia (Gilbert et al., 2006). *TRAK1* also modulates axonal mitochondria trafficking (van Spronsen et al., 2013; Ogawa et al., 2014). *TRAK1*

expression is elevated in colorectal cancer patients but the significance of this is unclear (Zhang et al., 2009; An et al., 2011).

6.4.18 HCFC1

Both of the male probands in Family 1 were found to be hemizygous for a *HCFC1* mutation (alanine to glutamic acid at protein position140). *HCFC1* (host cell factor C1) is located on the X chromosome and this mutation was predicted to be highly damaging by both PolyPhen and SIFT.

HCFC1, also known as VP-16 accessory protein, is involved in cell cycle control and regulation of transcription. Rare mutations in *HCFC1*, previously identified by WES, have been shown to cause transcriptional dysregulation leading to disruption of cobalamin metabolism. *HCFC1* does not encode an enzyme directly in the cobalamin pathway but is a transcriptional regulator of the enzymes involved. These mutations resulted in a severe neurological phenotype of intractable epilepsy and cognitive impairment (Yu et al., 2013).

6.5 Genetic homogeneity

An alternative to the 'family-based approach' is to analyse the WES data focusing on rare mutations common to a number of patients within the familial lupus nephritis cohort. Mutations with a MAF <1% in public databases (dbSNP, EVS and 1KG) were selected and their frequency within the lupus nephritis patients was compared with in-house WES cohorts at the Rockefeller University, namely Mendelian Susceptibility to Mycobacterial Disease (MSMD) patients (n=170) and a congenital asplenia cohort (n=52). A threshold p-value was chosen of 0.05/20,000 (number of genes = 2.50E-06). A selection of genes identified are shown in Table 6.9 however only mutations in *IGLV3-22* (immunoglobulin lambda variable 3-22) and *IFI44L* (interferon-induced protein 44-like) were exclusively present in the lupus nephritis cohort and not seen in the MSMD and congenital asplenia groups.

One missense mutation, (proline to histidine at protein position 62) in *IGLV3-22* was found in 9 lupus nephritis patients in our cohort and also in 6 unaffected family members. This mutation was predicted to be deleterious by both PolyPhen and SIFT. Three mutations in *IFI44L*, a type I interferon response gene were found in 5 lupus nephritis patients and in 1 unaffected family member in our study group. The first of these mutations was a heterozygous missense mutation (glycine to glutamic acid at protein position 209) (rs75931592) present in both probands of Family 7. Their unaffected mother did not have this mutation. This family was of African ancestry and the MAF in this population is 0.012. This variant was predicted to be highly damaging by both PolyPhen and SIFT. The second heterozygous missense mutation (serine to proline at protein position 193) (rs149221815) was present in both

probands of Family 2 and absent in their unaffected brother. This family was also of African ancestry and the MAF in this population is 0.001. The mutation was predicted to be highly deleterious by both PolyPhen and SIFT. The third *IFI44L* mutation (valine to alanine at protein position 62) was present in 1 proband and 1 unaffected sister in Family 6 but was not predicted to be damaging.

Table 6.9 Candidates identified by adopting a genetic homogeneity approach

	Mutations In SLE Cohort	**Number of SLE Patients**	**Mutations in Asplenia Cohort**	**Number of Asplenia Patients**	**Mutations in MSMD Patients**	**Number of MSMD Patients**
IGLV3-22	1	9	0	0	0	0
EHHADH	4	7	0	0	3	3
IFI44L	3	5	0	0	0	0
PUS7	3	6	0	0	2	2
C3orf67	5	6	1	1	2	2
MOCS2	3	6	0	0	5	5
CCR3	2	5	0	0	2	2

A comparison of rare variants (MAF<1%) present in the familial lupus nephritis cohort as compared to MSMD and congenital asplenia WES cohorts. *EHHADH*; enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase, *PUS7*; pseudouridylate synthase 7 homolog (S. cerevisiae), *C3orf67;* chromosome 3 open reading frame 67, *MOCS2*; molybdenum cofactor synthesis 2, *CCR3*; chemokine (C-C motif) receptor 3.

Chapter 7

Discussion

The purpose of this chapter is to:

1. Summarize the main findings of this research.
2. Discuss the limitations of this research
3. Provide concluding remarks and a discussion of perspectives.

7.1 Introduction

In this thesis, a multi-ancestral cohort of biopsy proven lupus nephritis patients have undergone genotype-phenotype correlations for common susceptibility polymorphisms in genes identified in SLE GWAS and, in select cases, for rare variants identified by WES.

Throughout this thesis, we progressed systematically from the initial clinical characterisation of the patient cohort to the generation of a list of novel genes for functional validation. We performed a number of demographic comparisons including between ancestries, gender, familial versus sporadic nephritis and juvenile versus adult onset disease. We explored autoantibody positivity, histology findings and renal outcomes in each of these categories and identified subgroups of patients with a severe clinical phenotype. We then correlated this clinical data with ImmunoChip findings in susceptibility polymorphisms involved in the HLA region, immune complex uptake, type I interferon signalling pathways, B and T cell signalling, and NFκB pathways. Finally, we used WES as a tool to identify rare but potentially causative mutations in cases of familial lupus nephritis.

7.2 Discussion of results

7.2.1 Discussion of clinical characterisation of lupus nephritis

The clinical characterisation of our lupus nephritis cohort has been extensively discussed in Chapter 2 and I will summarize some of the key findings here.

To recap, the ancestral background of our patient cohort was as follows: 41% European, 33% African, 13% South Asian, and 9% East Asian. East Asian patients had the earliest onset disease at 20.9 ±9.1 years, followed by South Asians at 23.9

±12.0 years and Africans at 26.8 ±10.7 years. Patients of European ancestry had the latest onset of disease at 27.1 ±11.9 years. 6% of patients of European ancestry have progressed to ESRD, while 12% of non-European patients have advanced renal impairment. This is consistent with reports in the literature of outcomes in patients with lupus nephritis in different ancestral groups (Dooley et al., 1997; Alarcon et al., 2001; Thumboo et al., 2001; Bastian et al., 2002; Johnson et al., 2006; Korbet et al., 2007).

Sixteen patients in our cohort had a first-degree family history of lupus nephritis including five sibling pairs. 69% of familial cases were African, 19% were South Asian and 13% were East Asian. No European patients in our study had a first degree relative with lupus nephritis. Familial cases were younger at diagnosis of nephritis, with 50% of juvenile onset as opposed to 22% in the sporadic onset group. Juvenile onset patients with a first degree family history of lupus nephritis in our study population were significantly more likely to develop advanced renal impairment than sporadic onset juvenile cases (25% versus 7%). In general, the literature supports this association of a more severe clinical phenotype with familial lupus nephritis (Freedman et al., 1997; Apenteng et al., 2006; Al-Mayouf et al., 2006). A large study of multiplex SLE families of European ancestry, however, did not demonstrate worse clinical outcomes compared to sporadic cases (Michel et al., 2001).

27% of patients in our cohort were diagnosed with nephritis before 18 years of age, 19% before the age of 16 years and 2% under the age of 10 years. This is consistent with the literature (Jimenez et al., 2003; Mina et al., 2010; Watson et al., 2012). In our study, juvenile onset patients were more likely to progress to ESRD than those with adult onset disease (16% versus 7%). It is well established that juvenile onset lupus nephritis patients tend to have a more aggressive disease course when

compared to those with adult onset disease (Font et al., 1998; Brunner et al., 2008; Sato et al., 2012). 57% of juvenile onset patients who progressed to ESRD in our study population were of African ancestry, 29% were of South Asian descent and 14% were European. This finding is consistent with a previous study that clustered juvenile onset SLE into mild, moderate and severe disease and demonstrated that 20% of European patients were in the severe cluster as compared to 51% of Asian and 41% of African patients (Levy et al., 2013).

In our cohort, males were diagnosed with lupus nephritis at a significantly younger age (21.3 ± 14.8 years) than their female counterparts (26.6 ± 10.2 years). We did not demonstrate a significant difference in progression to advanced renal impairment between genders with 9% of females and 12% of males reaching KDOQI stages 4 and 5. Some authors have observed an increased rate of progression to ESRD in male lupus nephritis (Specker et al., 1994; Carbone et al., 2002) while others have not demonstrated a difference in disease severity (Miller et al., 1983; Renau et al., 2012).

In our study, juvenile onset, familial cases of non-European ancestry had a particularly severe clinical phenotype. Several probands within these families have progressed to ESRD and are currently on dialysis or have already undergone renal transplantation. These patients and their unaffected relatives, where available, in addition to genotyping by ImmunoChip, have undergone WES to search for rare but highly penetrant variants that may have contributed to the development of their severe disease state.

7.2.2 Discussion of histopathological findings in lupus nephritis

The histologic variables examined in this research were CD68, as a general monocyte/macrophage marker, and the ISN/RPS classification of lupus nephritis. ISN/RPS classes I, II and V nephritis were found to have minimal glomerular CD68 infiltration and were, henceforth, excluded from our clinical analysis. We focused instead on the proliferative classes III, IV-G and IV-S. Class III nephritis had significantly less CD68 enrichment than classes IV-G and IV-S. We did not find a significant difference in glomerular CD68 cell count between class IV-G and IV-S. This is contrary to previous work by Hill et al, who showed that class IV-G nephritis had more glomerular monocytes/macrophages than class IV-S nephritis (Hill et al., 2005). A number of retrospective studies have shown significant clinical and morphological differences between classes IV-G and class IV-S. There is a lack of consensus, however, as to which class has a worse clinical outcome. In our study, of the patients who progressed to ESRD, 56% had class IV-G, 33% had class IV-S, and 11% had class III nephritis.

Glomerular CD68 count correlated with age at diagnosis of nephritis (r=-0.314, $^{**}p$=0.0096). A subgroup of patient with disease onset before age 10 years had particularly high CD68 counts. We found a strong positive correlation between CD68 with AI (r= 0.525, $^{****}p$<0.0001) and a negative correlation between CD68 and CI (r= -0.3128, $^{*}p$=0.021) indicating a role for CD68 cells in active inflammation. CD68 count was higher in familial nephritis (13.3 ± 8.0) than in sporadic cases (8.9 ± 6.8) but this did not reach statistical significance (p=0.07). As a continuation of this work, immunostaining of renal biopsies with CD16 is ongoing and will help decipher the functional heterogeneity of the mononuclear phagocytic system in lupus nephritis. Other markers are also being considered for

immunostaining including CX3CR1. In situ hybridization in renal tissue is being considered as it offers the prospect of examining a number of markers simultaneously at transcript level.

Correlation of glomerular CD68 count and ImmunoChip polymorphisms revealed some interesting observations. Patients who were homozygous for the risk allele in any of the three *ITGAM* polymorphisms studied tended to have low CD68 glomerular counts. The *ITGAM* rs1143679 minor allele is known to cause functional impairment of phagocytosis and adhesion (MacPherson et al., 2011; Rhodes et al., 2012). Low CD68 glomerular infiltration was also evident in carriers of the *RASGRP3* risk allele. *RASGRP3* has also been proposed to have a functional role in phagocytosis in macrophages (Botelho et al., 2009). This gene was originally associated with SLE in an East Asian GWAS and has not to date been replicated in European or African SLE patients (Han et al., 2009). No one in our cohort was homozygous for the *RASGRP3* risk allele. Patients who were heterozygous for the risk variant had predominantly membranous nephritis and, in those with proliferative nephritis, low glomerular CD68 immunostaining was seen. Two gene candidates from GWAS were associated with higher glomerular CD68 counts, *IKZF1* and those homozygous for the HLA rs9271366 risk allele. Patients with either of these risk variants tended to have juvenile onset nephritis.

The factors that determine glomerular CD68 infiltration remain unclear. From our research, age at diagnosis of nephritis and the influence of genetic polymorphisms may play a role. It is our intention to return to ImmunoChip and test if other polymorphisms, in addition to the SLE GWAS polymorphisms already tested in our study, are also associated with a high glomerular CD68 count.

7.2.3 Discussion of ImmunoChip results

We used the ImmunoChip to perform genotyping of known susceptibility polymorphisms associated with SLE in GWAS. A major drawback of this approach is that the ImmunoChip is designed for use in populations of European ancestry and is therefore less informative in other ancestral groups. Advantages of the ImmunoChip are that it is relatively inexpensive as compared to GWAS chips and that its use may allow clinical comparisons and collaborations with other research groups who have used the same tool.

GWAS have identified common variants but, due to their small effect sizes, these have only contributed a limited amount to the overall understanding of the heritability of SLE. Their discovery has, thus far, had little clinically meaningful impact on the ability to predict who will develop SLE, who will develop nephritis, who will have intractable disease and progress to ESRD and whether or not patients will be likely to respond to different treatment modalities.

In this thesis, susceptibility polymorphisms have been examined both individually and in aggregate where the effects of common variants were combined in a polygenic risk score. A discussion of each individual polymorphism has been provided in Chapter 4. In our cohort, we have found associations in European lupus nephritis patients with both of the HLA region polymorphisms studied, the *IRF5* rs2070197 polymorphism, three *ITGAM* variants, both *STAT4* polymorphisms examined, both *TNFAIP3* variants and with *TNFSF4*. Associations were found in African lupus nephritis patients with *ITGAM* rs1143679, *STAT4* rs7574865 and *ETS1*. No associations were seen in any ancestral group with *IRF7*, *IRF8*, *IRAK1*, *PTPN22*, *NCF2*, *IFIH1*, *BANK1*, *BLK*, *LYN* or *RASGRP3*, however lack of power in

this study cannot rule out an association of these susceptibility genes with lupus nephritis.

With regard to our polygenic risk score, European lupus nephritis patients tended to have the highest scores while African and East Asian patients tended to have lower scores. Two previous studies have found a higher polygenic risk score in juvenile onset SLE patients (Taylor et al., 2011; Webb et al., 2011). Our study did not reveal any convincing correlation between age at diagnosis of lupus nephritis and cGRS or wGRS in European, African or East Asian patients. South Asian juvenile onset patients in our cohort had a higher cGRS and wGRS than those of the same ancestry with adult onset disease.

In our polygenic risk score, we combined polymorphisms in genes functioning in a variety of different biologic pathways. An interesting alternative approach may be to devise risk scores for specific functional pathways. An interferon-related polygenic risk score, for example, could be devised by combining *IRF5/IRF7/IRF8/STAT4/IFIH1* risk alleles. An NFκB pathway polygenic score could be comprised of *IRAK1* and *TNFAIP3* risk alleles. A B-cell risk score could be compiled by combining *BLK, BANK1* and *LYN* polymorphisms. This approach may determine which biologic pathways are most affected in patient subgroups, potentially identifying pathways to target therapeutically.

It may be worthwhile to focus, in the future, on the patients in our cohort who have a low polygenic risk score. These individuals may represent interesting candidates in which WES could be used in order to seek an alternative genetic aetiology of their disease that does not involve the lupus susceptibility polymorphisms already known.

The associations between known susceptibility polymorphisms and lupus nephritis seen in our study were mainly in patients of European ancestry. As already mentioned above, polygenic risk scores were also highest in Europeans. The majority of variants tested in this research were identified in GWAS of European SLE patients and to a lesser extent in East Asian populations. Many of these variants have not been associated with African SLE. The paucity of GWAS data on patients of African ancestry is a significant limitation in lupus research in general as these individuals have more frequent and more severe disease. There is little known, therefore, about the genetic aetiology of lupus in patients of African ancestry and there is the possibility that, as yet, unidentified common variants may be present in these individuals. Rare variants almost certainly contribute, as *de novo* mutations are known to be up to three times more common in individuals of African ancestry than in European or Asian populations (Abecasis et al., 2012).

Further exploration of the Immunochip data in our cohort may include examining the frequency of polymorphisms on ImmunoChip in lupus nephritis patients as compared to ancestry matched healthy control data. To date we have focused on polymorphisms with known association with SLE, using this approach may identify new associations.

7.2.4 Discussion of WES results

The first step in our WES analysis was to filter the variants found in our study patients' data set against known polymorphisms in public databases such as dbSNP, EVS and the 1,000 Genomes Project. This narrowed down the list of candidates based on the assumption that any variant found frequently in the public filter set cannot be causative. With this in mind and considering the prevalence of lupus nephritis in the general population, we chose mutations where the MAF was <1% for homozygous mutations and where variants were absent from public data bases in the case of heterozygous mutations. WES of the lupus nephritis families in our cohort identified a large number of potential candidates. A promising short list from these candidates was then identified using pedigree information focusing on variants predicted to be damaging and by incorporating prior knowledge of the biologic pathways implicated in lupus.

Further key family members integral to analysis are being recruited for WES including the affected mother and unaffected father and brothers in Family 5. In Family 8, with a consanguineous background living in Pakistan, a cousin has been identified living in the United States who is reported to have a diagnosis of SLE. We are in the process of contacting this individual, obtaining further clinical details and DNA for WES if possible.

Mutations in genes involved in activation of Ras pathways (*RASGRF2, RASSF5, RIN1)* are potentially very interesting candidates for functional investigation. Recent literature suggesting that RASopathies may be associated with SLE and the previous identification of *RASGRP3* in East Asian GWAS adds credence to the candidates

identified in our study. In addition, all of these candidates were predicted to be highly deleterious.

Variants found in reactive intermediate genes *(GSR, NOS1, NDUFA13*) are also of considerable interest. These mutations are all predicted to be damaging and the literature supports their role in lupus pathogenesis. Mutations in two of these genes (*GSR, NDUFA13)* were found in one patient in our cohort who is of South Asian ancestry. The mutation in *NOS1* was found in two sisters with lupus nephritis of African descent which is of interest as polymorphisms in *NOS1* have been associated with SLE patients of African ancestry (Ramos et al., 2013).

Mutations in genes encoding the epsilon DNA polymerase complex (*POLE, POLE3*) are certainly potential candidates for functional testing given their role in DNA repair and histone folding. Both mutations were predicted to be deleterious however two unaffected sisters in Family 7 had the *POLE* variant which would argue against direct causation by this gene if apparently healthy individuals carry the mutation.

One complexity which could arise and must be taken into consideration is the possibility of incomplete penetrance where a given genetic mutation is not phenotypically expressed in all those who carry it.

Mutations in genes involved in ubiquitination (*USP36* and *UBR4)* are worthy of consideration for functional validation. The mutations identified were likely to be damaging and the evidence from the literature supports the importance of ubiquitination in lupus pathogenesis.

The compound heterozygous mutations in *DEFB119* found in Family 6 are also worthy of consideration for functional testing. The role of defensins in innate

immunity, NET formation and as a granulopoiesis-related marker makes this gene an attractive candidate for validation studies (Bennett et al., 2003; Ishii et al., 2005; Sthoeger et al., 2009; Vordenbaumen et al., 2010).

Mutations in genes encoding synaptotagmin-like proteins and chromodomain helicase DNA binding proteins were identified in multiple families within our cohort. A number of factors render these genes less attractive candidates for functional investigation. It is not immediately apparent how these genes would feature in lupus pathogenesis; however, this would not eliminate these candidates from the list for functional testing. It was noted that not all of these mutations are predicted to be deleterious by PolyPhen and SIFT. A number of diseases have already been associated with these genes which may argue against their role as causative genes in SLE.

Mutations in serine/threonine protein kinases (*MAPK3*, *MAP3K6, STK33)* were seen in a number of families in our study. Disappointingly the mutation in *MAPK3,* present in all three probands in Family 8 was not predicted to be damaging. A potentially deleterious mutation in *STK33* was seen in Family 2 in both probands but was also present in their unaffected brother. A further mutation in *STK33* in Family 5 was predicted to be damaging. Recently polymorphisms in *STK17A* were associated with SLE in a Brazilian study (da Silva Fonseca et al., 2013).

Mutations in *MACF1, COL6A5, DDR1* and *BOC* were present in more than one family with SLE but these mutations were not consistently predicted to be deleterious. In the case of *DDR1,* a role has been proposed for this gene in renal damage and inhibition of *DDR1* activity has also been shown to be protective against

renal damage in crescentic glomerulonephritis, a somewhat contradictory finding if one assumes we have identified a loss-of-function mutation (Kerroch et al., 2012).

One of the most interesting WES findings in our cohort was the discovery of compound heterozygous mutations in *TRAP1/DNASE1* in Family 5. Our current knowledge of lupus aetiology supports the functionality of these mutations with evidence both from murine models and monogenic lupus, with previous *DNASE1* and *DNASE1L3* mutations identified in SLE patients in Japan and Saudi Arabia (Yasutomo et al., 2001; Al-Mayouf et al., 2011). Disappointingly neither of the mutations identified in our study were predicted to be deleterious.

Candidate mutations in *IRF9* and *TLR9* are also of interest given the well-established role of endosomal TLRs and type I interferon pathways in the pathogenesis of SLE. The mutations were for the most part not predicted to be deleterious however.

Family 1 provided the most complete set of familial WES data as DNA was available for both affected sons and for their unaffected parents. This allowed for a more streamlined analysis. In this family, an autosomal recessive or an X-linked recessive model of inheritance looked most likely. With this in mind, mutations in *PTBP3* and *HCFC1* seem to be two interesting candidates for functional testing. Both affected sons were homozygous for the *PTBP3* mutation (which was predicted to be damaging) while the parents were both heterozygotes. The mutation in *HCFC1* which is located on the X chromosome could make sense in the context of Family 1 if the mother were an unaffected carrier.

The proband in Family 5 merits special mention given the constellation of deleterious mutations discovered in this individual by WES. This patient was the youngest and, phenotypically, the most severe in our cohort with his disease onset at

the age of 2 years and early progression to ESRD requiring renal transplantation. His mother also had lupus nephritis but with an older age of disease onset and a less severe clinical phenotype. The proband had mutations in two reactive intermediate genes; *GSR* and *NDUFA13*, two variants in ubiquitination genes; *UBR4* and *USP36*, and mutations in *STK33* and *RASSF5*. This same individual had the *IGLV3-22* mutation, in common with other patients in our cohort, as identified by the genetic homogeneity approach. Mutations in other potentially interesting genes such as the autophagy gene, *ATG13*, and in *ACIN1* (apoptotic chromatin condensation inducer) were also found in this patient, both of which were predicted to be damaging. He also had the functionally interesting though not predicted to be damaging mutation in *TRAP1/DNASE1*. A high composite genetic risk score analysed from GWAS polymorphisms in ImmunoChip was present in this patient. As mentioned previously, his parents and unaffected brothers are being recruited for WES. This should help elucidate which of these interesting mutations are contributing to the disease phenotype in this fascinating case. Recent data has suggested that every human genome harbours significant numbers of loss-of–function variants including stop codons, frameshift mutations and large deletions and that the load of potentially damaging rare variants carried by and tolerated by apparently healthy individuals is significantly larger than was anticipated (MacArthur et al., 2012). It is important to consider the possibility of epistasis in which the effect of one gene or genetic mutation depends on the presence of one or more modifier genes.

IGLV3-22 and *IFI44L* were identified using the genetic homogeneity approach. Both of these genes are potentially interesting candidates in the context of SLE pathogenesis. *IFI44L* expression is increased in the synovium of SLE patients and a genome-wide methylation study of CD4+ T cells from lupus patients showed

significant hypomethylation of *IFI44L* amongst other interferon-regulated genes (Nzeusseu Toukap et al., 2007; Coit et al., 2013). There is no specific literature on *IGLV3-22* in the context of SLE although a gene involved in VDJ recombination may certainly be of interest in SLE given the important role of autoantibodies in the disease. The *IGLV3-22* and *IFI44L* mutations were also seen in unaffected family members, however, weakening the claims for these mutations to be causative in lupus pathogenesis.

A useful tool under development that may allow us to narrow down the list of candidate genes of interest is the GDI (Gene Damage Index). The GDI is a genome-wide estimation of mutational damage per base pair per individual for all protein-coding genes in humans with respect to all other human genes. The GDI can be applied to the analysis of NGS data, as less damaged genes are more likely to underlie rare monogenic diseases (as yet unpublished, courtesy of Dr Yuval Itan, Professor Jean-Laurent Casanova, Rockefeller University, New York).

Functional validation of the top candidates is being planned to determine how a variant may have a meaningful impact in the disease processes underpinning SLE.

7.2.5 Outline of plan for functional validation of gene candidates:

- Initial verification of candidate mutations by Sanger sequencing
- Validate at the transcript level
- Validate at the protein level (Western blot/mass spectrometry)
- Modelling to predict changes in protein structure
- Consider animal model, for some candidates models are already in existence, if not consider a knock-in model for the mutation of interest.
- Consider RNA-Seq (RNA sequencing) or eQTL (expression quantitative trait loci) to complement WES data.
- Devise functional assay for candidate gene mutations, for example serum DNase activity in the patient with *DNASE1/TRAP1* mutation.
- PBMCs, serum and further renal biopsy tissue are available for the majority of patients. There is also ethical approval to return to the patients/unaffected relatives for repeat sampling.
- Opportunity to collaborate with other research groups who are using WES in juvenile and familial SLE cases who may have found mutations in the same gene candidates or biologic pathways.

7.3 Limitations of this research

One of the main limitations of this research is the number of lupus nephritis patients studied, in particular with regard to common variants with low effect sizes. This was further limited by the need for population stratification given the difference in allelic frequencies in different ancestral groups and the need to avoid bias. The East and South Asian lupus nephritis patients comprised a particularly small group. It was not our intention, however, to replicate a GWAS but, rather, to examine known susceptibility polymorphisms in patients with lupus nephritis who are seen in every day clinical practice. Despite this, we did still find associations in several GWAS hits such as polymorphisms in the HLA region, *ITGAM*, *TNFAIP3* and *STAT4*. Other candidate gene polymorphisms identified in GWAS were not associated with lupus nephritis in our cohort, although, given the lack of power in the study we cannot state that these are not associated with disease. In addition, there are polymorphisms in candidate genes that have been associated with SLE but that have not been covered by this research including *ATG5*, *UBE2L3*, *IL10*, *TYK2*, *ELF1*, *TNIP1* and *WDFY4*.

Another limitation of this research is that autoantibody results and renal biopsies material were not available for all patients recruited. Autoantibody availability was as follows; ANA 99% (n=162), anti-dsDNA 98% (n=160), anti-Ro 93% (n=152) and 92% (n=151) for anti-RNP and anti-Sm. Paraffin-embedded renal biopsy tissue was available from 77% (n=126) of the patients recruited to the study. When immunostaining was analysed, slides with < 7 glomeruli present were excluded, leaving 117 biopsies for CD68 analysis and 114 biopsies for ISN/RPS classification. Urinary protein-creatinine ratio, AI and CI results were not available for all study

participants. We have reported if a patient ever tested anti-dsDNA antibody positive but did not collect information regarding antibody titres. Anti-C1q antibody information was not collected.

To minimise the effects of immunosuppressive medication on renal biopsy findings, biopsies were traced back to the time of the patients' original diagnosis of lupus nephritis (n=107) or, where this was not possible biopsies taken at the onset of a new nephritis flare before induction immunosuppression was commenced were obtained (n=19). Many patients were likely to be taking some degree of immunosuppression at the time of biopsy but this has not been adjusted for and is a potential cause of bias. Collecting the data regarding details of immunosuppression at time of biopsy proved to be difficult due to the retrospective nature of this research and the fact that patients were attending a number of different hospitals.

With regard to the WES results, key family members have not yet been sequenced, for example in Family 3, Family 5 and Family 8. This constitutes a considerable limitation to the data so far in that the list of potential candidates for functional testing cannot be narrowed down to a reasonable number without these individuals. This issue is now being addressed and recruitment of additional family members is ongoing.

We reported the clinical outcomes in our lupus nephritis patients using the NKF KDOQI scoring system as a measure of the degree of chronic kidney disease. We did not use any of the validated lupus activity or damage scores such as the BILAG, SELENA-SLEDAI or SLICC which may be considered a limitation. The retrospective nature of the study prohibited collection of this data which was further

compounded by the fact that the patients were recruited from a number of different centres under the care of several different physicians.

7.4 Concluding remarks and future perspectives

SLE susceptibility genes identified in GWAS have certainly contributed to our understanding of the aetiopathogenesis of the disease. The 'missing heritability' of SLE remains somewhat of a mystery, however. Interestingly, GWAS hits may represent markers for the true underlying causative variants. *IFIH1*, for example, was initially associated with SLE in a large replication study of European SLE patients and recently mutations in *IFIH1* have been identified as causing interferonopathy (Cunninghame-Graham et al., 2011; Rice et al., 2014).

In our study, a number of mutations in genes involved in activation of Ras pathways were discovered. This is interesting as *RASGRP3*, a functional partner of these genes, was associated with SLE in a GWAS of East Asian patients (Han et al., 2009) and RASopathies were recently proposed as novel monogenic conditions predisposing to the development of SLE (Bader-Meunier et al., 2013). Polymorphisms in reactive intermediate genes were previously associated with SLE in African patients. In our cohort, rare mutations were identified in *GSR, NOS1* and *NDUFA13*. The most striking of these was a homozygous mutation in *GSR* that encodes for a stop codon early in the protein that was predicted to be highly deleterious and to have significant functional consequences.

When using WES to discover novel variants in SLE, we focused on patients with an extreme clinical phenotype, in particular those with familial disease and who had juvenile onset of disease. With increasing accessibility to WES and reducing costs, research groups are using this technology to sequence large groups of unrelated patients to search for residual unidentified common variants of disease. Furthermore, WGS is becoming increasingly available and will provide information on mutations

throughout the entire genome rather than focusing exclusively on the exome. This approach will reveal much larger candidate mutation lists and the challenge will be to identify the true causative variants.

Several other parameters need to be taken into consideration in the context of the genetics of SLE which we have not explored or discussed in our study. Gene-gene and gene-environment interactions undoubtedly play a role in SLE pathogenesis. Epigenetic alterations such as DNA methylation and histone modifications are also likely to contribute to the development of disease and may explain some of the 'missing heritability' of SLE.

We look forward to the functional validation of the candidates identified in this research and sincerely hope that these will deepen our understanding of the disease processes underlying SLE. Five further families with clustering of two or more individuals with SLE and eight juvenile sporadic onset male lupus nephritis patients are currently undergoing WES (Appendices 21-23). It will be interesting to see what new variants emerge and if mutations in the candidate genes uncovered in this data set will also be identified in these families.

For too long, SLE therapy has relied on general immunosuppressive agents with non-specific effects to which many patients remain refractory. It is essential that more targeted therapies, focusing on the root causes of the disease, are developed in order to improve outcomes for patients. Studies of patients with monogenic causes of SLE may be particularly instructive. The phenotype of the Trex1 knock out mouse, for example, has been salvaged using reverse transcription inhibitors and trials in AGS patients are soon to begin (Beck-Engeser et al., 2011). While monogenic causes of SLE are rare, they provide extremely useful insights into our understanding

of the disease pathways underlying SLE. The monogenic causes of SLE identified to date have helped fill in the gaps in our knowledge of lupus pathogenesis and helped to elucidate the roles of complement pathways, apoptosis and aberrant TLR and type I interferon signalling. Therapeutic agents targeting these biologic pathways are in development.

Appendix 1: ISN/RPS classes in different ancestral background in lupus nephritis

	European (n=45)	African (n=36)	South Asian (n=16)	East Asian (n=11)
Class I	2% (n=1)	3% (n=1)	0%	0%
Class II	7% (n=3)	0%	13% (n=2)	0%
Class III	20% (n=9)	36% (n=13)	31% (n=5)	27% (n=3)
Class IV-G	29% (n=13)	22% (n=8)	25% (n=4)	9% (n=1)
Class IV-S	33% (n=15)	17% (n=6)	31% (n=5)	27% (n=3)
Class V	9% (n=4)	22% (n=8)	0%	36% (n=4)

Appendix 2: ISN/RPS classes of lupus nephritis in familial and sporadic disease

	First Degree Familial (n=10 biopsies available)	Sporadic (n=99 biopsies available)	*p*-value	OD	95% CI
Class I	0%	2% (n=2)	-	-	-
Class II	0%	5% (n=5)	-	-	-
Class III	40% (n=4)	26% (n=26)	0.458	1.87	0.489 to 7.17
Class IV-G	30% (n=3)	22% (n=22)	0.693	1.50	0.358 to 6.29
Class IV-S	20% (n=2)	30% (n=30)	0.720	0.58	0.115 to 2.87
Class V	10% (n=1)	14% (n=14)	1.0	0.68	0.0792 to 5.75

Appendix 3: ISN/RPS classes of lupus nephritis in juvenile and adult onset disease

	Juvenile (n=30 biopsies available)	Adult (n=84 biopsies available)	*p*-value	OD	95% CI
Class I	0%	2% (n=2)	-	-	-
Class II	0%	6% (n=5)	-	-	-
Class III	30% (n=9)	26% (n=22)	0.787	1.13	0.454 to 2.84
Class IV-G	23% (n=7)	25% (n=21)	0.764	0.86	0.324 to 2.29
Class IV-S	30% (n=9)	27% (n=23)	0.889	1.07	0.428 to 2.66
Class V	17% (n=5)	13% (n=11)	0.764	1.26	0.399 to 3.97

Appendix 4: ISN/RPS classes of lupus nephritis in male and female disease

	Male (19 biopsies available)	Female (95 biopsies available)	*p*-value	OD	95% CI
Class I	0%	2% (n=2)	-	-	-
Class II	5% (n=1)	4% (n=4)	1.0	1.26	0.133 to 12.0
Class III	26% (n=5)	27% (n=26)	1.0	0.95	0.310 to 2.89
Class IV-G	32% (n=6)	23% (n=22)	0.436	1.53	0.521 to 4.50
Class IV-S	26% (n=5)	28% (n=27)	1.0	0.90	0.295 to 2.74
Class V	11% (n=2)	15% (n=14)	1.0	0.68	0.141 to 3.28

Appendix 5: ISN/RPS class and autoantibody profile in lupus nephritis

Autoantibody	Class I (n=1)	Class II (n=5)	Class III (n=30)	Class IV-S (n=32	Class IV-G (n=27)	Class V (n=16)
ANA	100%	100%	100%	100%	95%	94%
Anti-dsDNA	100%	60%	80%	84%	75%	56%
Anti-Ro	0%	20%	43%	34%	29%	38%
Anti-Sm	0%	0%	17%	28%	29%	31%
Anti-RNP	0%	40%	43%	38%	38%	56%

Appendix 6: Homozygous and X-linked recessive WES candidates of interest in Family 1

Gene	Mutation	PolyPhen	SIFT	Function
Homozygous Mutations				
PTBP3 (polypyrimidine tract binding protein 3)	P7L Chromosome 9	High	High	Binds RNA and regulates cell differentiation
TRAK1 trafficking protein, kinesin binding 1	E/- 630, non frameshift deletion Chromosome 3	-	-	Regulation of endosome-to-lysosome trafficking
X-linked recessive				
DMD (Dystrophin)	E2013G	Moderate	High	Duchenne and Becker muscular dystrophies
GNL3 (Guanine nucleotide binding protein-like 3)	L/- 59 non frameshift deletion	-	-	Role in stem cell proliferation
HCFC1 Host cell factor C1	A140E	High	High	Chromatin modulation Located on Xq28, adjacent to IRAK1, MECP2

The full list of mutations found in Family 1 is available at http://brahma.rockefeller.edu/polyweb

Appendix 7: Heterozygous WES candidates of interest in Family 1

Gene	Mutation	PolyPhen	SIFT	Function
XIRP2 (xin actin-binding repeat containing 2)	Stop codon Q/* 372 Chromosome 2	-	-	Role in actin binding
CENPF (centromere protein F)	Stop codon Q/* 2580 Chromosome 1	-	-	Component of nuclear matrix during G2 phase of interphase
CGB2A1 (secretoglobin, family 2A)	Frameshift deletion DSI/- 86 Chromosome 11	-	-	Androgen binding
CX3CR1 chemokine (C-X3-C motif) receptor 1	V143M Chromosome 3	Moderate	High	Fractalkine receptor
VSIG2 V-set and immunoglobulin domain 2	N125I Chromosome 11	High	High	SNPs associated with SLE (Kariuki et al., 2010)
TLR9 Toll like receptor 9	A1185S Chromosome 3	Low	High	Endosomal TLR
IRF9 interferon regulatory factor 9	R102H Chromosome 14	Low	Low	IFN signalling pathway
SYTL3 synaptotagmin-like 3	D25H Chromosome 6	High	High	Role in vesicle trafficking

The full list of mutations found in Family 1 is available at http://brahma.rockefeller.edu/polyweb

Appendix 8: WES candidates of interest in Family 2

Gene	Mutation	PolyPhen	SIFT	Function
Homozygous Mutations				
GMNC (geminin coiled-coil domain containing)	E28D Chromosome 3	Low	Low	Regulator of DNA replication
Compound Heterozygote				
COL6A5 (collagen, type VI, alpha 5)	T299P rs61734775 MAF 0.002	Low	Low	Roles in organisation of tissue architecture and cell adhesion
	Stop codon, R/*1563 rs145841466 MAF 0.002	-	-	
Heterozygous Mutations				
STK33 serine/threonine kinase 33	K512N Chromosome 11	High	High	Serine/threonine protein kinase
RASGRF2 Ras protein-specific guanine nucleotide-releasing factor 2	R640C Chromosome 5	High	High	Nucleotide exchange factor, activating RAS/RAC1

The full list of mutations found in Family 2 is available at http://brahma.rockefeller.edu/polyweb

Appendix 9: WES Compound Heterozygous Mutations of Interest in Family 3

Gene	Mutation	PolyPhen	SIFT	Function
IRF9 interferon regulatory factor 9	V222F Chromosome 14	Low	Low	IFN signalling pathway
	A258E Chromosome 14			
MLPH melanophilin	E323K rs144852340 MAF 0.01 Chromosome 2	High	High	Role in melanosome transport
	E363V rs61736277 MAF 0.01 Chromosome 2	High	High	
MACF1 microtubule-actin crosslinking factor 1	R389Q rs140119894 MAF 0.024 Chromosome 1	High	High	Facilitates actin-microtubule interactions
	V1692I Chromosome 1	Low	Low	

The full list of mutations found in Family 3 is available at http://brahma.rockefeller.edu/polywe

Appendix 10: WES Heterozygous Mutations of Interest in Family 3

Gene	Mutation	PolyPhen	SIFT	Function
ATP8B4 (ATPase, class I, type 8B, member 4)	Stop codon G/* 315 Chromosome 15	-	-	Role in phospholipid transport in the cell
C4A (complement component 4A)	A1221D Chromosome 6	High	High	Classical activation pathway
ICAM2 (intercellular adhesion molecule 2)	R151S Chromosome 17	Moderate	High	Intracellular adhesion molecule
USP36 (ubiquitin specific peptidase 36)	V409L Chromosome 17	High	High	Transcriptional repressor
ATP6AP1 (*ATPase, H+ transporting, lysosomal accessory protein)*	V1361	High	High	Transporter activity
RALGAPB (Ral GTPase activating protein)	G577A	High	High	GTPase activator
DDR1 (discoidin domain receptor tyrosine kinase 1)	Q38E Chromosome 6	Low	Low	Regulates β1 integrin interactions with fibrillar collagen

The full list of mutations found in Family 3 is available at http://brahma.rockefeller.edu/polyweb

Appendix 11: WES Homozygous and Compound Heterozygous Mutations of Interest in Family 4

Gene	Mutation	PolyPhen	SIFT	Function
Homozygous Mutations				
BOC BOC cell adhesion associated, oncogene regulated	P828R rs150200848 MAF 0.030 Chromosome 3	Low	Low	Mediates cell-cell interactions between muscle precursor cells
TRPC7 transient receptor potential cation channel, subfamily C, member 7	D686Y rs184057936 MAF 0.004	Low	Low	Nil of note
Compound Heterozygous Mutations				
HSPG2 heparan sulfate proteoglycan 2	S137N rs62642527 MAF 0.018	High	High	Component of glomerular basement membrane
	P197T Splicing variant	-	-	Perlecan protein,
ITGA2 integrin, alpha 2	T892P rs116473481 MAF 0.012	Moderate	Low	Integrin, associated with bleeding disorder, platelet-type 9
	Variant in UTR	-	-	

The full list of mutations found in Family 4 is available at http://brahma.rockefeller.edu/polyweb

Appendix 12: WES Heterozygous Mutations of Interest in Family 4

Gene	Mutation	PolyPhen	SIFT	Function
NOS1 nitric oxide synthase 1	G50V Chromosome 12	High	High	Synthesize nitric oxide from L-arginine
RIN1 Ras and Rab interactor 1	R257W Chromosome 11	High	High	RAS binding protein
DHX36 DEAH (Asp-Glu-Ala-His) box polypeptide 36	L364M Chromosome 3	High	High	ATP-dependent RNA helicase
PRRC2B proline-rich coiled-coil 2B	R995C Chromosome 9	High	High	Unknown function

The full list of mutations found in Family 4 is available at http://brahma.rockefeller.edu/polyweb

Appendix 13: WES Homozygous Mutations of Interest in Family 5

Gene	Mutation	PolyPhen	SIFT	Function
GSR (glutathione reductase)	Stop codon E/* 32 Chromosome 8	-	-	Reduces glutathione disulfide (GSSGG) to the sulfhydryl form GSHH
DDR1 (discoidin domain receptor tyrosine kinase) *DDR1 mutation in Family 3	R119Q Chromosome 6	Moderate	Low	Regulates β1 integrin interactions with fibrillar collagen.
IRAK1 (interleukin-1 receptor-associated kinase 1)	R521H X Chromosome	Low	Low	IRAK1 SNPs associated with SLE in multiple ancestral groups.
IGLV3-22 (immunoglobulin lambda variable 3-22)	P62H	High	High	See Genetic Homogeneity Section

The full list of mutations found in Family 5 is available at http://brahma.rockefeller.edu/polyweb

Appendix 14: WES Compound Heterozygous Mutations of Interest in Family 5

Gene	Mutation	PolyPhen	SIFT	Function
TRAP1 **(overlap with *DNASE1*)** TNF receptor-associated protein 1	R650Q Chromosome 16	Low	High	*DNASE1* monogenic
	T65K Chromosome 16	Low	Low	
UBR4 ubiquitin protein ligase E3 component n-recognin 4	C933F Chromosome 1	High	High	Part of the chromatin scaffold in the nucleus.
	L4122V Chromosome 1	Moderate	Low	
CBS cystathionine-beta-synthase	V80M Chromosome 21	High	High	Conversion of homocysteine to cystathionine
	R45W Chromosome 21	Moderate	High	
MACF1 microtubule-actin crosslinking factor 1	R1070Q Chromosome 1	Low	Low	Actin-microtubule interactions
	Q2980H Chromosome 1 rs147777128 MAF 0.002	High	Low	

The full list of mutations found in Family 5 is available at http://brahma.rockefeller.edu/polyweb

Appendix 15: WES Heterozygous Mutations of Interest in Family 5

Gene	Mutation	PolyPhen	SIFT	Function
HIRA HIR histone cell cycle regulation defective homolog A	Stop codon W162* Chromosome 22	-	High	Transcriptional regulator
MAP3K6 mitogen-activated protein kinase kinase kinase 6	Frameshift deletion TG/- 640	-	-	Serine/threonine protein kinase
STK33 serine/threonine kinase 33	I306T Chromosome 11	Moderate	High	Serine/threonine protein kinase
ATG13 autophagy related 13	M359I	Low	Low	Required for autophagosome formation and mitophagy
SYTL2 synaptotagmin-like 2	intronic	-	-	Regulatory role in functioning of lysosome-related organelles
USP36 ubiquitin specific peptidase 36	G468V	Moderate	High	Transcriptional repressor
RASSF5 Ras association (RalGDS/AF-6) domain family member 5	E200Q	High	Low	Regulation of integrin medicated adhesion, and Ras induced apoptosis

COL6A5 collagen, type VI, alpha 5 *Mutation in *COL6A5* in Family 2	F680V	High	Low	Roles in organisation of tissue architecture and cell adhesion
ACIN1 apoptotic chromatin condensation inducer	R146H	Moderate	High	Induces apoptotic chromatin condensation after activation by caspase-3
NDUFA13 NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	R81Q	High	High	Subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase

The full list of mutations found in Family 5 is available at http://brahma.rockefeller.edu/polyweb

Appendix 16: WES Compound Heterozygous & X-linked Mutations in Family 6

Gene	Mutation	PolyPhen	SIFT	Function
Compound Heterozygous Mutations				
DEFB119 defensin, beta 119	A33V rs116939471 MAF 0.031	Low	Low	Immune defence against microbial infection
	T70N Chromosome 20	Moderate	High	
FAT3 FAT atypical cadherin 3	C356Y rs142950989 MAF 0.028 Chromosome 11	Low	Low	Nil known
	T70N Chromosome 11	High	High	
X-linked Mutations				
SYTL5 Synaptotagmin-like 5	non frameshift deletion K/- 577	-	-	Regulatory role in functioning of lysosome-related organelles
EFHC2 EF-hand domain (C-terminal) containing 2	L/F 197	Low	Low	Association with epilepsy

The full list of mutations found in Family 6 is available at http://brahma.rockefeller.edu/polyweb

Appendix 17: WES Heterozygous Mutations in Family 6

Gene	Mutation	PolyPhen	SIFT	Function
POLE polymerase (DNA directed), epsilon, catalytic subunit	I238F	High	High	Involved in DNA repair
BOC BOC cell adhesion associated, oncogene regulated	D79V	Low	Low	Mediates cell-cell interactions between muscle precursor cells
IL12RB1 interleukin 12 receptor, beta 1	G594E	Low	Low	Part of IL-12 receptor complex

The full list of mutations found in Family 6 is available at http://brahma.rockefeller.edu/polyweb

Appendix 18: WES Homozygous Mutations of interest in Family 7

Gene	Mutation	PolyPhen	SIFT	Function
SESTD1 SEC14 and spectrin domains 1	S96C rs147276383 MAF 0.01 Chromosome 2	Low	Low	Primary docking protein directing membrane turnover and assembly of the transient receptor potential channels
MKL1 megakaryoblastic leukemia (translocation) 1	M166T Chromosome 22	Low	Low	Role in transducing signals from the cytoskeleton to the nucleus
MAP10 microtubule-associated protein 10	T47A rs12094630 MAF 0.006 Chromosome 1	Moderate	High	Regulation of cell division; promotes microtubule stability
TFAP2A transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	G/A rs35008125 MAF 0.02 Chromosome 6	High	High	Transcription factor
TULP4 tubby like protein 4	V1084I rs34559793 MAF 0.02 Chromosome 6	Low	Low	Associated with cleft palate

The full list of mutations found in Family 7 is available at http://brahma.rockefeller.edu/polyweb

Appendix 19: WES Homozygous Mutations in Family 8

Gene	Mutation	PolyPhen	SIFT	Function
MAPK3 mitogen-activated protein kinase 3	G9D Chromosome 16	Low	Low	Serine/threonine kinase
KIAA0556	V452I rs200420515 MAF 0 Chromosome 16	Low	Low	Nil known
ADCY7 adenylate cyclase 7	A480V rs61731915 MAF 0.002 Chromosome 16	Moderate	High	Catalyses the formation of cyclic AMP from ATP
CHD9 Chromodomain helicase DNA binding protein 9	P2114R Chromosome 16	High	High	Role in chromatin remodelling
FAM129C family with sequence similarity 129, member C	A215T Chromosome 19	High	Low	Associated with CLL

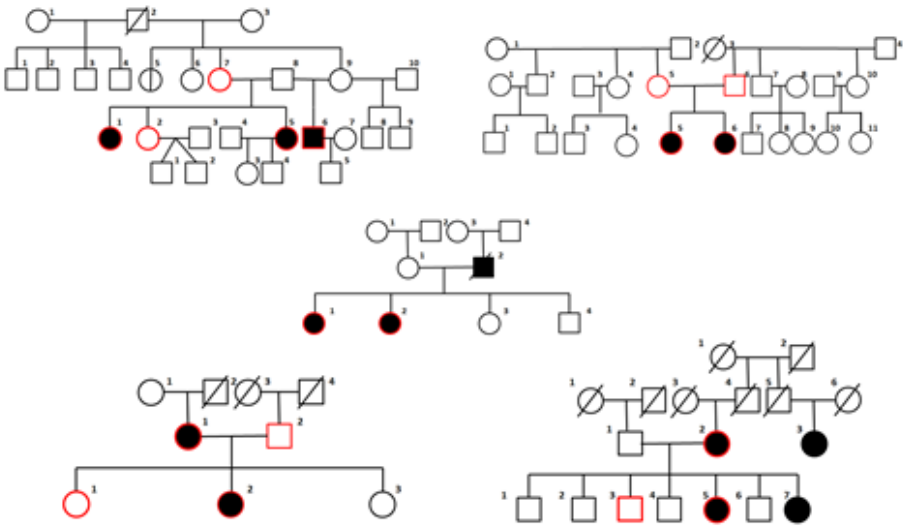
The full list of mutations found in Family 8 is available at http://brahma.rockefeller.edu/polyweb

Appendix 20: WES Compound Heterozygous and Heterozygous Mutations of Interest in Family 8

Gene	Mutation	PolyPhen	SIFT	Function
Compound Heterozygous Mutations				
MACF1 microtubule-actin crosslinking factor 1	Y8C Chromosome 1	-	High	Facilitates actin-microtubule interactions
	T3736N Chromosome 1	High	Low	
HEATR1 HEAT repeat containing 1	F1411L Chromosome 1	Moderate	High	Involved in nucleolar processing of pre-18S ribosomal RNA
	N610S Chromosome 1	Low	Low	
Heterozygous Mutations				
POLE3 polymerase (DNA directed), epsilon 3, accessory subunit	V69L Chromosome 9	High	High	Histone-fold protein

The full list of mutations found in Family 8 is available at http://brahma.rockefeller.edu/polyweb

Appendix 21: Multiplex families currently undergoing WES



Red outline indicates DNA is available

Appendix 22: Multiplex families currently undergoing WES

Subject Identifier	Age at diagnosis	Gender	Clinical Features
F9-A Proband (III-5)	27	F	Class IV-G
F9-B Proband (III-6)	24	M	End-stage renal failure secondary to lupus nephritis
F9-C Proband (III-1)	20	F	Cutaneous involvement only
F9-D Unaffected (III-2)	-	F	No SLE features
F9-E Unaffected (?) (II-7)	-	F	Primary biliary cirrhosis, inflammatory arthritis
F10-A Proband (III-5)	16	F	Class IV-S lupus nephritis
F10-B Proband (III-6)	19	F	Antiphospholipid syndrome, non-renal lupus
F10-C Unaffected (II-6)	-	M	No SLE features
F10-D Unaffected (II-5)	-	F	No SLE features
F11-A Proband (III-1)	20's	F	Cutaneous/musculoskeletal SLE
F11-B Proband (III-2)	20's	F	Cutaneous/musculoskeletal SLE
F12-A Proband (III-2)	25	F	Class V nephritis
F12-B Proband (II-1)	40's	F	Discoid lupus musculoskeletal involvement
F12-C Unaffected (III-1)	-	F	No SLE features
F12-D Unaffected (II-2)	-	M	No SLE features
F13-A Proband (IV-5)	18	F	Class IV nephritis severe skin involvement
F13-B Proband (III-2)	57	F	Cutaneous/musculoskeletal Antiphospholipid syndrome
F13-C Unaffected (IV-3)	-	M	No SLE features

Appendix 23: Male juvenile onset (sporadic cases) currently undergoing WES

Subject Identifier	Ancestry	Age at diagnosis	Class of nephritis	Autoantibody Profile
SLE 18	South Asian	10	Class IV-S	ANA, anti-dsDNA, anti-Ro
SLE 29	European	17	Class IV-G	ANA, anti-dsDNA
SLE 31	European	7	Class IV-G	ANA, anti-dsDNA
SLE 54	African	11	Class III	ANA, anti-dsDNA, anti-Ro, anti-Sm, anti-RNP
SLE 85	African	15	Class IV-G	ANA, anti-dsDNA, anti-Ro
SLE 106	European	11	Class IV-S	ANA, anti-dsDNA, anti-Ro
SLE 122	South Asian	14	Class IV-G	ANA, anti-dsDNA, anti-Ro
SLE 158	African	16	Class V	ANA, anti-dsDNA
SLE 165	European	11	Class III	ANA, anti-dsDNA

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